GENETIC INVESTIGATIONS ON FERTILITY RESTORATION TO THE S-TYPE CYTOPLASMIC MALE STERILITY IN MAIZE (*ZEA MAYS* L.)

By

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Dedicated to my beautiful Cas.

Together we once found happiness in the dreams we shared...

In dreams, we now seek to be together.

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Cytoplasmic male sterility (CMS) is a maternally inherited inability to shed viable pollen. Nuclear restorer of fertility (*Rf*) genes affecting the S-type sterile inducing cytoplasm of maize are gametophytically expressed. Therefore, the genotype of the pollen grain with respect to the *Rf* gene determines fertility. The most commonly occurring restorer gene for the S-type cytoplasm is *Rf3*. This gene has been located on the long arm of chromosome 2 (2L). Several other CMS-S *Rf* genes have been identified and located to unique positions throughout the maize genome. Conventionally, the allele responsible for fertility restoration has been assumed to be the functional, or dominant, form of the gene. However, in order to assess the dominant-recessive interaction it is necessary to observe the expression of both alleles in the appropriate diploid tissue.

The dominant *Rf3* allele was ascertained by using both phenotypic and genetic inheritance tests. An ability to track the inheritance of *Rf3* alleles required the identification of closely linked markers. Selected RFLP loci were tested for linkage with fertility restoration among a population of BC1 progeny. The *whp* and the *bnl17.14* loci were found to be 6.4 cM proximal and distal to *Rf3* respectively.

To express *Rf3* in diploid pollen, tetraploid CMS-S maize plants were generated using the elongate (*el*) mutant. Plants homozygous *el/el* produced the unreduced 2n female gametes needed for fertilization with 2n pollen from the tetraploid line W23 conversion. Microscopic examination of 2n pollen shed by CMS-S plants with the genotype *Rf3/Rf3/rf3/rf3* showed a segregation ratio of 5 normal : 1 aborted grains indicating the restoring *Rf3* allele was functional. Conclusive evidence for dominance of the restoring allele was obtained by showing co-transmission of both alleles through the pollen from these plants.

A new *Rf* gene for the S-type cytoplasm was discovered in the maize inbred line VA20. In reference to the standard *Rf3* gene, inheritance data indicated that this new restorer was gametophyically expressed. Southern analysis with the *whp* and *bnl17.14* RFLPs showed this gene was not linked to *Rf3* located on 2L.

CHAPTER 1
INTRODUCTION

Cytoplasmic male sterility (CMS) is the maternally inherited inability to shed viable pollen and is known to occur extensively throughout the plant kingdom (Edwardson, 1970; Laser and Lersten, 1972). The CMS trait has proven value both as an efficient method to produce F1 hybrid plants and as a means to investigate the interaction between the nucleus and cell organelles, particularly the mitochondria. The majority of the maize male sterile inducing cytoplasms can be classified as either T (Texas), C (Chuarra) or S (USDA) type. Distinctions may be made by differences in the fertility of restorer (*Rf*) genes, mode of restoration, and mitochondrial DNA restriction profiles.

Restoration by the *Rf3* gene and the S1 and S2 mitochondrial plasmids are among the unique features which differentiate the S-type cytoplasm. Fertility restoration by *Rf3* is gametophytic; i.e. the phenotyp4e of each pollen grain is determined by its genotype. Approximately equal quantities of functional and aborted pollen grains are therefore produced by CMS-S plants heterozygous for the *Rf3* gene. This semi-sterile phenotype is a striking contrast to the fully fertile phenotype displayed by the sporophytically restored CMS-T and CMS-C maize plants which may be either homozygous or heterozygous for their restorer gene(s). The *Rf3* allelic designations had arbitrarily been assigned dominance (*Rf3*) for the restored and recessive (*rf3*) for the aborted phenotypes. The purpose of this research was to discern the dominant allele, i.e. functional form, of the *Rf3* gene. This included the identification of mapped molecular markers closely linked proximal and distal to *Rf3* and the generation of CMS-S tetraploid (4n) plants. These plants were assayed for their pollen phenotype. An additional genetic test used these characterized plants to fertilize another 4n maize plant, thereby transmissability of the *Rf3* alleles through the 2n pollen was tested. In an unrelated investigation, another gametophytically expressed gene capable of restoring fertility to the maize S-type cytoplasm was discovered.

CHAPTER 2
LITERATURE REVIEW

Cytoplasmic Inheritance in Higher Plants

Higher plant genetics may be divided into Mendelian and extrachromosomal types of inheritance. Extrachromosomal inheritance refers to the cytoplasmic transmission of genes encoded by the chloroplast (cpDNA) and mitochondrial (mtDNA) genomes. Non-Mendelian segregation seen as differences between progeny from reciprocal crosses or somatic segregation genetically distinguishes cytoplasmic from the nuclear inherited traits.

Somatic segregation of alleles can be seen following natural biparental organelle inheritance or induced protoplast fusion. Either event results in heteroplasmic cells containing organelles from both maternal and paternal origin. The apparently unstable heteroplasmic cytoplasms subsequently assort their organelles randomly during mitotic divisions and eventually develop homoplasmic cell lines (Smith, 1989).

Much of the information regarding organelle inheritance and the mechanisms for controlling transmission comes from studying the chloroplasts. This is largely due to the molecular restriction fragment length polymorphism (RFLP) markers and the number of relatively easily identifiable mutants. Included among chloroplast mutant phenotypes are chlorophyll deficiencies (Kirk and Tilney-Bassett, 1978), tentoxin sensitivity (Schadler et al., 1976), triazine resistance (Souza Machado, 1982), streptomycin, and lincomycin resistance (Maliga, 1984). The difficulty in obtaining similar information concerning mitochondrial inheritance may in part be attributed to the comparatively few non-lethal and easily identifiable genetic markers. Cytoplasmic male sterility (CMS) (Hanson and Conde, 1985; Levings and Pring, 1979; Newton, 1988) and the maize non-chromosomal stripe (*ncs*) mutant (Newton and Coe, 1986) have provided strong correlative evidence for maternal inheritance of the mitochondria in angiosperms. Organelle inheritance among the gymnosperms has been shown to be more variable. An unusual inheritance pattern was observed in *Pinus taeda* when progeny received cpDNA from the paternal parent and mitochondrial DNA (mtDNA) from the maternal parent (Neale and Sederoff, 1989). In contrast, *Sequoia sempervirens* D. paternally transmitted both mtDNA and cpDNA to its progeny (Neale et al., 1994). Paternal transmission of chloroplast DNA (cpDNA) has been demonstrated with restriction fragment length polymorphisms (RFLPs) in other gymnosperms (Neale et al., 1986; Wagner et al., 1987). Earlier electron microscopy studies by Chesnoy and Thomas (1971) had suggested organelles were paternally transmitted in several species within the gymnosperms. Owens and Morris (1991) later presented cytological evidence supporting predominant paternal inheritance of organelles in *Pseudotsuga menziesii*. The *Ginkgoales* and the *Cycadales* appear to provide distinctive examples within the gymnosperms which inherit organelles maternally (Whatley, 1982).

The angiosperms, on the other hand, generally show cytoplasmic organelle inheritance to be maternal. Exceptions occur in those species which also exhibit some degree of biparental inheritance (Sears, 1980). The extent of biparental plastid transmission in *Pelargonium* has been shown to be influenced by the combined maternal nuclear and plastid genotypes (Tilney-Bassett, 1970), whereas in *Oenothera* it is reported to be largely influenced by the organelle genome (Chiu et al., 1988; Corriveau and Coleman, 1990), and the nuclear genotype appears to be the major influence in *Petunia* (Cornu and Dulieu, 1988; Derepas and Dulieu, 1992). Compared to the chloroplasts, reports indicating biparental or paternal inheritance of mitochondria (Soliman et al., 1987) and mitochondrial associated DNA (Erickson et al., 1989) is rare. The prohibitive mechanism(s) by which paternal mitochondria are excluded from sexual transmission are unknown. The correlation between the mitochondria and the CMS phenotypes, and the ability of nuclear genes to suppress the sterile inducing effects of the cytoplasm can be important resources for gaining information on the inheritance and function of these organelles in gametogenesis.

Characteristics of Cytoplasmic Male Sterility

CMS has traditionally been described as the maternally inherited inability to shed viable pollen and is known to occur extensively throughout the plant kingdom (Edwardson, 1970; Laser and Lersten, 1972). For some of the most important agronomic crops this type of male sterility has economic value as an efficient method to produce superior F1 hybrids. Examples of crops where the possible utilization of

CMS has been examined include, maize, wheat, rice, sunflower, tobacco, carrot, potato, onion, sorghum, pearl millet, barley and sugar beet (Duvick, 1959; Hanson and Conde, 1985; Kaul, 1988). Plants with an otherwise normal appearance may display characteristic differences in their CMS phenotype. Male sterility resulting from changes in floral morphology has been described in *Daucus* (Tanno-Suenaga et al., 1991), *Nicotiana* (Clayton, 1950), *Solanum* (Grun et al., 1977), and *Brassica* (Pearson, 1972). A commonality amongst the *Nicotiana*, *Solanum*, and *Brassica* genera is that CMS may arise from an incompatibility associated with alloplasmic (interspecific) hybridizations. CMS may also result from abortion at various stages during microsporogenesis. Laser and Lersten's (1972) review on CMS in angiosperms compiled descriptive characteristics of anthers, tapetal cells, microspore development and cytological observations at the time of abortion for 29 genera. The phenotypic diversity of the CMS phenomenon among these genera suggests they may result from an assortment of unique nuclear-cytoplasmic interactions.

Accumulated evidence pertaining to CMS has implicated the mitochondria in these nuclear-cytoplasmic interactions. The first evidence was uncovered by Levings and Pring (1976) when they noted a correlation between alterations in mtDNA but not cpDNA restriction digest patterns of maize with the Texas type (CMS-T) sterile inducing cytoplasm. Similar observations have been made in *Nicotiana* (Asahi et al., 1988) and in sorghum (Conde et al., 1982). CMS associated mtDNA rearrangements have been reported in a wide range of other plant species including *Petunia* (Boeshore et al. 1985) *Brassica* (Erickson et al., 1986) *Helianthus* (Crouzillat et al., 1987) Ogura radish (Makaroff and Palmer, 1988) and sugar beet (Mikami et al., 1986). In some species, further molecular investigations have resulted in the association of CMS with specific mtDNA sequences, mRNA transcripts, and translation products (Dixon and Leaver, 1982; Hanson and Conde, 1985; Kofer et al., 1991; Laughnan and Gabay-Laughnan, 1983; Levings and Pring, 1979; Nair, 1993; Williams and Levings, 1992).

In addition to the molecular data there is cytological and biochemical evidence which supports the hypothesis that mitochondria play a crucial role in the occurrence of CMS. Ultrastructural studies have shown mitochondrial abnormalities within the tapetal cells of CMS-petunia (Bino, 1985), rape (Grant, 1986), radish (Kaul, 1988), sorghum (Overman and Warmke, 1972), and maize (Lee et al., 1979; Warmke and Lee, 1977) which correlated with aberrant development during the process of male gamete formation. Ultrastructure studies by Warmke and Lee (1977) showed mitochondrial abnormalities occurred shortly after meiosis within the tapetum and middle layer of anthers from maize plants with the CMS-T cytoplasm. Biochemical information related to changes in mitochondrial function has been obtained primarily from the petunia and maize T-type CMS. Distinctive differences in DNA synthesis in the tapetal cells (Liu et al., 1987), mitochondrial ATP export (Liu et al., 1988), and alternative oxidase activity (Connett and Hanson, 1990) have each been observed between the male-sterile inducing and the non-sterile cytoplasms in petunia. Maize plants with the T-type CMS are susceptible to the fungus *Bipolaris maydis* race T which causes the disease known as Southern Leaf Blight. Kono and Daly (1979) purified BmT pathotoxin produced by the fungus. CMS-T mitochondria exposed to this toxin were shown to undergo both biochemical and morphological changes. These changes were seen in respiration (Holden and Sze, 1989; Matthews et al., 1979), organelle swelling (Miller and Koeppe, 1971), small molecule leakage (Holden and Sze, 1984), and as the uncoupling of oxidative phosphorylation from electron transport (Bednarski et al., 1977). BmT toxin specificity was later connected to the CMS-T unique URF13 polypeptide which is encoded by the mitochondrial gene T-*urf13* (Dewey et al., 1987; Dewey et al., 1988; Forde et al., 1978; Forde and Leaver, 1980; Levings, 1990; Wise et al., 1987). Because the URF13 polypeptide is unique to CMS-T, BmT toxin sensitivity may be used to discern this cytoplasm.

Generally, CMS cytoplasms are classified according to differential responses to specific nuclear restorer of fertility genes (Ahokas, 1982; Beckett, 1971; Kaul, 1988), here on referred to as *Rf*. These genes act to preclude the expression of the male sterile phenotype. CMS-T (Texas), CMS-C (Chuarra) and CMS-S (USDA) are the three major male sterile cytoplasmic groups which have been identified for maize using this criteria (Beckett, 1971; Briggle, 1957; Duvick, 1965). Chromosomal location of the maize *Rf* genes have shown them to be nonallelic (Duvick et al., 1961; Laughnan and Gabay, 1978; Sisco, 1991; Snyder and Duvick, 1969). The mode by which these genes restore each cytoplasm also differs. CMS-T and CMS-C maize plants display a male fertile phenotype when the appropriate *Rf* genes are expressed sporophytically. These systems differ in that a fully male fertile phenotype for CMS-T plants can be attained only when the two dominant genes *Rf1* and *Rf2* are present, whereas in most cases the CMS-C plants may require only the dominant *Rf4* gene (Kheyr-Pour et al., 1981; Vidakovic, 1988).

The unexpected occurrence of nonsegregating backcross progeny from male parents presumably heterozygous for a *Rf* gene led to the discovery of a fundamentally different fertility restoration system than the sporophytic systems previously described. Buchert (1961) conducted inheritance experiments to investigate this phenomenon and was able to deduce that the fertility restoration process was conditioned by a single gene and took place at a time when the cytoplasms of the microspores were separated, i.e. in the developing pollen grain. Therefore, maize plants with the CMS-S cytoplasm are restored to fertility by the gametophytically expressed *Rf3* gene, and the nonrestoring allele of this gene can be transmitted only through the female gamete. The pollen phenotype of heterozygous *Rf3* CMS-S maize plants viewed under magnification shows approximately 50% normal and 50% aborted grains. This phenotype is consistent with Buchert's model for a gametophytically expressed *Rf* gene. Gametophytic expression of a CMS restorer gene has also been reported in rice by Shinjyo (1969). Research by Lee et al. (1980) provided information on the cytological events associated with pollen abortion in the CMS-S plants. They found that the male gametes which do not carry the restoring *Rf3* allele degenerated just prior to reaching maturity. This is a much later stage than that which occurs with T- and C-type cytoplasms. Because it is gametophytically expressed, the *Rf3* alleles were arbitrarily designated; *rf3* for the aborted and *Rf3* for the starch filled pollen phenotypes.

The correlation between the mitochondria and CMS provides additional factors by which cytoplasmic groups can be characterized and classified. Susceptibility to the pathotoxin BmT and the pesticide methomyl have been used to identify maize carrying the CMS-T cytoplasm (Laughnan and Gabay-Laughnan, 1983; Levings, 1990; Pring and Lonsdale, 1989). Using molecular techniques the mitochondrial genome itself has been analyzed in several species. Agarose gel fractionated restriction endonuclease digested mtDNA fragments result in banding profiles. These profiles have been powerful aides in research which 1) identified a rearrangement associated with fertility restoration in *Phaseolus vulgaris* (Mackenzie et al., 1988), 2) demonstrated protoplast-fusion (Boeshore et al., 1983; Perl et al., 1990), 3) investigated organelle inheritance in sexual hybridizations (Conde et al., 1979), 4) identified unique bands associated with the maize *ncs* mutant phenotype (Newton and Coe, 1986), 5) compared differences between mtDNA from fertile and male sterile inducing cytoplasms (Borck and Walbot, 1982; Kemble et al., 1980; Lee et al., 1989; Pring and Levings, 1978), and 6) classified cytoplasmic genotypes (Bailey-Serres et al., 1986; Sisco et al., 1985). The first major maize CMS-S subgroups were categorized by Sisco et al. (1985) based on mtDNA banding profiles in conjunction with the response to nuclear fertility restoration. Investigations concerning the structure and organization of the mitochondrial genome have been performed with RFLP analysis. By using this technique it has been possible to study the stoichiometry of mtDNA as it may be related to genome diversity (Small et al., 1987; Spruill, et al., 1980), construct genetic and RFLP maps of the mitochondrial genome (Brears and Lonsdale, 1988; Fauron et al., 1989), study mtDNA recombination events (Brears and Lonsdale, 1988; Fauron et al., 1990), and perform sequence homology experiments (Stern and Lonsdale, 1982). Changes in mtRNAs and translation products may be detected when there are differences in mitochondrial genome organization and stoichiometry. Unique CMS related mtRNAs and polypeptides have been studied in a number of species including; petunia (Wolf-Litman et al., 1992; Young and Hanson, 1987), maize (Boutry et al., 1984; Dewey et al., 1986; Dewey et al., 1987; Forde and Leaver, 1980; Forde et al., 1978; Forde et al., 1980; Kennell et al., 1987; Rocheford et al., 1992), and sorghum (Bailey-Serres et al., 1986; Dixon and Leaver, 1982). These unique transcripts and polypeptides were found to be encoded in the main high-molecular-weight (hmw) mitochondrial genome and in plasmid like molecules present in the mitochondria (Nair, 1993).

One of the definitive properties of the maize S-type sterile inducing cytoplasm is the presence of the mitochondrial plasmids now known as S-1 and S-2 (Pring et al., 1977). Previous to this discovery, Laughnan and Gabay (1975) had proposed that an episomal system was responsible for the maize CMS-S type male sterility. Because the S-1 and S-2 molecules fit this model, subsequent research has been focused on how they might be causal agents of this CMS phenomenon.

S-1 and S-2 were shown to be double stranded linear plasmid-like DNAs (Pring et al., 1977) approximately 6.4 and 5.4 kilobases (kb) long, respectively (Levings and Sederoff, 1983; Paillard et al., 1985). Significant sequence similarity between the terminal ends was indicated by the formation of stem loop structures as seen in certain electron microscopy experiments (Weissinger et al., 1982). Sequence analysis later revealed that the molecules had identical terminal inverse repeats (TIRs) that were 208 base pair (bp) long (Levings and Sederoff 1983; Paillard et al., 1985). Moreover, a large region of homology exists between S-1 and S-2 which extends beyond one TIR (Kim et al., 1982; Levings et al., 1983). Encoded within this additional 1262 bp region is the 1017 bp unidentified reading frame URF2 (Levings and Sederoff, 1983; Paillard et al., 1985). URF3 (2787 bp) and URF4 (768 bp) are encoded in the S-1 unique DNA (Paillard et al., 1985), and URF1 is located within S-2 unique DNA (Levings and Sederoff, 1983). Fertile revertant plants which carried only the S-1 and S-2 sequences integrated into the hmw mtDNA showed the loss or significant reduction in URF1 and URF2 transcripts (Schardl et al., 1985; Traynor and Levings, 1986). Products produced by *in vitro* translation experiments have shown no correlation between URF1 or URF3 and the fertility status of CMS-S maize plants (Zabala and Walbot, 1988; Zabala et al., 1987). Therefore, it would seem that URF1 and URF3 encode products responsible for some function other than directly causing the sterile CMS phenotype.

Apparent autonomous replication as well as structural similarity to other linear DNA molecules not associated with CMS further supports this suggestion that S-1 and S-2 sequences may be necessary for some function(s) other than inducing the male sterile phenotype. The linear S-1, S-2 molecules have been shown to have a protein attached to their 5' ends (Kemble and Thompson, 1982). This resembles the DNA-protein association seen in some adenoviruses (Rekosh et al. 1977) and *Bacillus* phages

(Harding et al. 1978; Ito 1978; Yoshikawa and Ito, 1981) which is believed to be involved in replication. The TIRs and replication mechanism as visualized in electron microscopy studies (Sederoff and Levings, 1985) also suggest a relationship to the adenoviruses and *Bacillus* phages. Furthermore, independent replication of S-1 and S-2 as indicated by their approximately five-fold molar excess relative to the (hmw) mtDNA in most CMS-S cytoplasms (Laughnan and Gabay-Laughnan, 1983) is in accordance with this proposed relationship. The RU cytoplasm of some maize species indigenous to South America may be identified by the presence of the R-1 and R-2 mitochondrial plasmids (Weissinger et al., 1982). A high degree of molecular similarity exists between these molecules and S-1 and S-2, yet the RU cytoplasms are fertile.

The possibility that S-1 and S-2 play some role other than encoding the gene(s) directly responsible for CMS is still being explored. CMS-S male sterility has been shown to be unstable, with fertility arising from either nuclear (Laughnan and Gabay, 1973) or cytoplasmic changes (Singh and Laughnan, 1972). While this instability is detrimental for hybrid seed production, it provides a viable experimental approach to uncover the cause of the sterility. Initial molecular characterizations of CMS-S cytoplasmic reversions to fertility revealed the loss of free S-1 and S-2 plasmids and a reorganization of the hmw mtDNA (Laughnan et al., 1981; Levings et al., 1980; Schardl et al., 1985). Ishige et al. (1985) and Escote-Carlson et al. (1988), however, reported that cytoplasmic revertants with the Wf9 nuclear background retained free S-1 and S-2 plasmids. This finding, together with the evidence presented above, strongly suggests that their presence as free plasmids in the mitochondria is not sufficient to account for the CMS phenotype.

Sequences homologous to S-1 and S-2 have been detected in the mitochondrial chromosomal DNA from S-type and N-type cytoplasms (Kemble and Mans, 1983; Levings et al., 1980; Lonsdale et al., 1981; Spruill et al., 1980; Thompson et al., 1980) and alterations in this DNA is a commonality among all CMS-S cytoplasmic revertants studied thus far (Escote-Carlson et al., 1988; Ishige et al., 1985; Kemble and Mans, 1983; Levings et al., 1980; Schardl et al., 1985). By characterizing the S-homologous regions in S-type cytoplasms, Schardl et al. (1984) were able to demonstrate recombination between the free plasmids and the hmw mtDNA. This recombination was seen as the linearization of a high proportion of the circular mitochondrial genome. Additional recombinational events were proposed to give rise to internal S-plasmid sequences in the hmw mtDNA. Ultimately, these internal sites may have provided the homologous sequences required for the possibility of recombinational integration of the free plasmids into the hmw mtDNA. The inability to detect S-1 and S-2 plasmids concurrent with a reorganization of the mtDNA was the basis for this proposal.

The discovery that S-1 and S-2 were retained in cytoplasmic revertants with the Wf9 nuclear background implied that CMS-S male sterility was determined by a mechanism other than that which had previously been proposed. Still common to all the examined CMS-S cytoplasmic revertants was the observation that the chromosomal mtDNA underwent a reorganization which consistently involved the S-homologous regions. Reorganizations were also seen when mtDNA from CMS-S revertants was probed with a clone of the *coxI* gene, thus, indicating other sequences within the genome may be involved in CMS (Escote-Carlson et al., 1988; Leaver et al., 1985). The possibility that the S-type CMS is due to a change in mitochondrial genome organization is consistent with findings in other CMS systems (Nair, 1993).

Because the nuclear genome has a variety of effects on the mitochondrial genome, it is plausible that there is more than one manner by which it influences the occurrence and expression of CMS. The relative molar amounts of the S-1 and S-2 molecules (Laughnan and Gabay-Laughnan, 1983), the stoichiometries of specific mtDNA restriction fragments (Leaver et al., 1985), and the patterns of mtDNA reorganization observed in cytoplasmic revertants to fertility (Small et al., 1988) have each been shown to be affected by the nuclear genome, although the significance of each with regard to CMS expression is unclear. A more clear influence of the nuclear genome was seen in the frequency and fertility of nuclear and cytoplasmic CMS-S revertants (Laughnan et al., 1981). Moreover, direct effect of the nucleus on the expression of CMS is seen with the *Rf* genes.

As described above, *Rf* genes have been identified by their ability to override the cytoplasmic effects to result in male fertility. The means by which the fertile phenotype is attained is currently unknown. Considering the presently available information on CMS types, pollen development, and a variety of associations with other physiological phenomenon, it would be reasonable to expect that not all *Rf* genes perform in the same basic manner. The timing of pollen abortion, which may occur at all stages during spermatogenesis, is characteristic for many species (Laser and Lersten, 1972). The possibility exists that *Rf* genes may have unique effects on steps in a biological pathway (Izhar, 1977). Conversely, a common effect which may be expected of certain inhibitors is also possible. The complexity of the male fertility restoring systems suggests that neither possibility need be exclusive.

Factors involved in overriding the effects of a male sterile inducing cytoplasm vary among the systems. First, male fertility may result from either sporophytically or gametophytically expressed nuclear *Rf* genes. Plants which are heterozygous for sporophytic restorers display a fully fertile phenotype, whereas in gametophytic restoration, heterozygotes are partially fertile and do not transmit the nonrestoring allele through the male gametes. Maize and rice are two species where both gametophytic and sporophytic restoration systems have been documented (Beckett, 1971; Raj and Virmani, 1988; Shinjo, 1969). Second, the number of major *Rf* genes needed to restore fertility is not a constant between species and may be variable within species. Several examples of what may be considered multi-faceted, nuclear restorer systems are included in a review by Hanson and Conde (1985). The variable responses to *Rf* genes can generate confusion in the attempts to characterize cytoplasms and assess the genetics of fertility restoration. For example, maize plants carrying the C-type cytoplasm most often undergo fertility restoration in the presence of a single *Rf* gene (*Rf4*). However, in some hybrid combinations, the same cytoplasm required two and three complimentary *Rf* genes for full fertility restoration (Josephson et al., 1978; Vidakovic, 1988). Moreover, the genetic interaction and expression between multiple

Rf genes has been reported to differ depending on the nuclear background (Maan, 1985; Raj and Virmani, 1988). Third, the male fertile phenotype of CMS plants is subject to influences external to the major *Rf* genes. Differences in fertility restoration of some CMS cytoplasms by the appropriate *Rf* genes have been observed in certain hybrid combinations. This significant variation in fertility is usually attributed to nuclear background and/or environmental effects. At the molecular level, comparisons among specific hybrids have shown the nuclear genome to affect several aspects of mitochondrial biology. In their experiments designed to examine the effects of the nuclear genome on mitochondria, Kennell et al. (1987) reported changes in mtRNA transcription patterns separate from fertility restoration; Bailey-Serres et al. (1986) noted that the products of *in organello* protein synthesis were affected, and Sisco et al. (1984) found differences in the recoverable amount of mitochondrial dsRNA associated with the maize LBN cytoplasm. CMS plants have shown temporal and spatial variation in the fertile phenotype itself. This variation has often been attributed to the interaction of environmental factors such as temperature, moisture, and daylength, with different nuclear backgrounds or modifier genes (Briggle, 1957; Blickenstaff et al., 1958; Duvick and Noble, 1978; Edwardson, 1955; Josephson et al., 1978; Lemke et al., 1985; Tracy et al., 1991). In maize, nuclear background and environmental factors seemed to affect fertility of the S-type cytoplasm more than either the T- or C-type cytoplasms (Briggle, 1957; Josephson et al., 1978). Perhaps this is a consequence of the lateness in pollen abortion in the CMS-S system (Lee et al., 1980). That CMS-S fertility is subject to nuclear modifiers was also implied by Josephson et al. (1978)

when they suggested that many maize inbreds contained weak CMS-S restorer genes. The complexity and unpredictability of the nuclear genome in effecting male fertility restoration has been further demonstrated in wheat (Maan, 1992a; Maan, 1992b). The species cytoplasm-specific (scs) nuclear genes; *Rf* (male fertility-restoring), *Fi* (male fertility-inhibiting), and *Vi* (plant vigor-restoring) are known to produce different phenotypes depending on the nucleocytoplasmic hybrid combination in which they are expressed. One might consider the *Fi* and *Vi* genes to be modifiers of fertility restoration. Wheat which displayed a restored male fertile phenotype without the benefit of any major *Rf* genes has been developed. Fertility of these plants is likely due to the interaction of modifiers such as *Fi* and *Vi*. While CMS in wheat occurs as a consequence of interspecific hybrid nuclear-cytoplasmic incompatibility, it is nevertheless conceivable that similar nuclear modifier genes are responsible for fertility restoration in the intraspecifically derived form of CMS. From these examples, it is clear that the expression of male fertility in CMS systems is not a genetically simple trait and is further confounded by being subject to external influences.

Gametophytic Gene Expression

Gametophytic gene expression occurs at various stages throughout the process of sexual reproduction in higher eukaryotes. This has been demonstrated by both genetic inheritance and tissue specific mRNA studies. Gametophytic factors (*Ga*) affecting pollen tube growth and consequently fertilization in maize were reported by Manglesdorf and Jones (1926). The self-incompatibility gene systems which serve to prevent inbreeding in plants are designated either gametophytic or sporophytic, depending on the type of interaction between the male gamete and the female sporophytic tissue. In both cases, however, the gametophytic expression of some factor in the pollen is recognized by the female somatic tissue (East, 1932; East and Mangelsdorf, 1925). Some of the species in which these systems have been recognized and investigated are *Trifolium* (Townsend, 1965), *Lolium* (Thorogood and Hayward, 1991), *Brassica*, and several solanaceous species (Haring et al., 1990). Much of the research on gametophytically expressed genes involves mature pollen grains and their ability to effect fertilization. Alternatively, the restorer of fertility gene *Rf3* is illustrative of a gametophytically expressed gene required for pollen development to reach completion. This requirement is specific to CMS-S maize plants which demonstrates a critical interaction between the nucleus and the cytoplasm.

Mutations and Their Effects on Meiosis

Cells normally undergo two divisions during the course of meiosis resulting in gametes with one-half of the nuclear genome. In the first division homologous chromosomes pair and genetic recombination can take place. These events are followed by the disjunction of the paired homologs and their migration to opposite poles of the cell. Because this results in two distinct groups of n chromosomes this is known as a reductional division ($2n \rightarrow n$). In the second division, known as an equational division, the sister chromatids separate to form two pairs of 1n gametes. Gametogenic cells which fail to normally complete either the first or second division can result in the production of unreduced gametes (2n). Depending upon genic recombination, the disruption of the first meiotic division (known as First Division Restitution, FDR) versus an incomplete second meiotic division (known as Second Division Restitution, SDR) in heterozygous plants results in the production of genetically distinct gametes. This has been of particular interest in potato breeding research which involved the manipulation of species with diploid (2n) genomes to generate tetraploid (4n) plants (Hermsen, 1984; Jongedijk, 1985; Ramanna, 1979; Stelly and Peloquin, 1986).

Genome manipulations may be performed with the aid of several different techniques. In maize, Randolph (1932) utilized temperature treatments to proembryos to induce chromosome doubling. Rhoades (1956) observed that 2n eggs were produced in varying proportions by maize plants homozygous recessive for the elongate (*el/el*) gene. As a means to develop maize autotetraploids for breeding purposes, Alexander (1957) proposed a technique to utilize the elongate gene to genetically induce chromosome doubling of diploid inbreds. Subsequent investigations have shown that SDR is the mechanism principally responsible for the 2n eggs produced by this meiotic mutant (Nel, 1975).

Both 1n and 2n eggs but only 1n pollen are produced on *el/el* plants (Rhoades and Dempsey, 1966). A 2n X 1n fertilization results in an imbalance between maternally and paternally derived chromosomes in the endosperm which results in a shriveled kernel (Lin, 1984). The same phenotype occurs with a 1n X 2n fertilization. These characteristic phenotypes allow for the identification and recovery of the normally reduced or unreduced gametes from the mixed ear. However, because a low frequency of aneuploidy has been reported to occur (Rhoades and Dempsey, 1966), the selected progeny should be subjected to another verifying assay such as a testcross.

CHAPTER 3
RFLP LINKAGE WITH *RF3* AND IDENTIFICATION OF A NEW NATURALLY OCCURRING RESTORING GENE

Introduction

Cytoplasmic male sterility (CMS) is the maternally inherited inability to shed viable pollen and is known to occur in approximately 140 species, 47 genera and 20 families of plants (Edwardson, 1970; Laser and Lersten, 1972). Experimental evidence pertaining to the expression of the CMS phenotype has shown a strong correlation with the mitochondria. Changes in either the mitochondrial DNA (mtDNA) (Fauron et al., 1987; Laughnan et al., 1981; Mackenzie et al., 1988; Schardl et al., 1985; Wise et al., 1987) or a favorable nuclear-cytoplasmic interaction (Hanson and Conde, 1985) can result in the production of functional pollen by CMS plants. Nuclear genes which are believed to overcome or suppress a sterile inducing cytoplasm are generally referred to as restorers of fertility (*Rf*). Maize *Rf* genes and their mode of restoration distinguish three major groups of CMS known as T (Texas), C (Charrua) and S (USDA) (Beckett,1971; Buchert, 1961; Duvick, 1965; Duvick et al., 1961; Gracen and Grogan, 1974; Laughnan and Gabay-Laughnan, 1983). Correspondingly, the mitochondrial genomes for each group display characteristic features (Borck and Walbot, 1982; Forde et al., 1980; Kemble et al., 1980; Laughnan and Gabay-Laughnan, 1983; Levings and Pring, 1976; Pring and Levings, 1978; Sisco et al., 1985).

Rf3 is a major nuclear gene responsible for fertility restoration of the maize CMS-S cytoplasm. *Rf3* is gametophytically expressed, consequently, it is the genotype of the pollen grain itself which determines functionality or abortion (Buchert, 1961; Lee et al., 1980). Pollen examined under low power microscopy reveals a semi-fertile phenotype for plants heterozygous for *Rf3*, i.e. approximately 50% of the pollen grains have a normal, starch-filled appearance while the remaining 50% have a non-staining, collapsed phenotype. Fully fertile and male-sterile phenotypes are characteristic of the *Rf3* homozygotes. The inheritance of male fertility (Duvick, 1965) and pollen phenotypes either singly or in combination with chromosomal aberrations (Burnham, 1982) were utilized to conduct mapping experiments for CMS-S *Rf* genes (Laughnan and Gabay, 1978). Results from these tests showed tight linkage, indicating allelism, of naturally occurring CMS-S restorers in maize inbreds. These "standard" *Rf3* genes mapped to the long arm of chromosome 2 (2L).

CMS-S *Rf* genes which mapped to new chromosome locations have also been identified (Laughnan and Gabay, 1973; Laughnan and Gabay, 1978). The sources of these new *Rf* genes were unexpected male fertiles found among the progeny of inbred CMS-S plants crossed with their corresponding isogenic nonrestoring counterpart (maintainer) (Laughnan and Gabay, 1973; Laughnan and Gabay, 1978). Like *Rf3*, these CMS-S *Rf* genes are gametophytically expressed (Laughnan and Gabay, 1975). With one exception the newly arisen *Rf* gene(s), distinct from the naturally occurring *Rf3* gene, were further characterized by their pleiotropic effects which included zygotic lethality, reduced kernel size, or the loss of fertility restoration capabilities in specific genotypic combinations (Laughnan and Gabay 1975).

The identification and distinctiveness of these additional restorers suggests the possibility that the CMS-S male sterile phenotype may result from any number of currently unidentified lesions in the mitochondrial genome. Therefore, it could logically follow that each mitochondrial lesion affects a specific step in the developmental pathway of the male gametophyte or the efficiency with which the mitochondria function in energy production. Further investigations, including physiological and molecular characterizations of all the CMS-S *Rf* genes, could result in basic information on plant reproduction and some of the roles of nuclear-mitochondrial interactions.

Advancement in genetic study and ultimately molecular cloning of CMS-S fertility restorers would be greatly aided by more precise localization of the genes than previously accomplished with cytogenetic techniques. The use of translocation and inversion heterozygotes in the CMS-S *Rf* linkage studies has several disadvantages. Among these is the requirement to determine the percentages of aborted pollen grains due to the non-restoring *rf* allele and to the chromosomal aberration. Also, the degree of male-fertility can be affected by the external variables temperature, daylength and moisture (Blickenstaff et al., 1958; Briggle, 1957; Josephson et al., 1978; Marshall et al., 1974; Tracy et al., 1991; Vincent and Woolley, 1972), such that male-sterile plants can be falsely identified without appropriate genetic test crossing. This research reports the identification of maize chromosome 2 RFLPs (Burr and Burr, 1991;

Franken et al., 1991) which are linked to *Rf3*. Use of these linked markers in conjunction with male-fertility data shows that the naturally occurring restorer from the inbred VA20 genome is a new *Rf* gene which is not genetically linked to the standard *Rf3* gene.

Materials and Methods

Plant materials

The USDA (S) cytoplasm and its subgroups are presented in parenthesis in association with genomic designations. Subgroups of the S cytoplasm are designated (B), (TC), (VG), (CA), (LF), (OY) and (NT) (Sisco et al., 1985). The normal cytoplasm is designated (N).

The restored inbreds KY21(S) and CE1(VG) and the non-restored inbreds B73(S) and B73(N) were provided by J. Laughnan and S. Gabay-Laughnan (U. of Illinois). The restored inbreds VA20(CA), VA20(B) and VA20(TC) and the nonrestored inbreds W182BN(CA), W182BN(LF), NYD410(CA), NYD410(OY), PA884p(CA) and PA884p(NT) were provided by the Cornell Cytoplasm Bank (Cornell University). The nonrestored inbreds WF9(S) and WF9(N) were provided by P. Chourey (U. of Florida). Linkage experiments between CMS fertility restoration and previously mapped RFLP loci (Burr and Burr, 1991; Franken et al., 1991) described below were conducted with the following populations: the 47 BC1KY21^{W182BN} progeny examined were generated from the cross W182BN(CA) X [W182BN(CA) X KY21(S)]; the 37 BC1VA20^{W182BN} and 40 BC1VA20^{B73} tested progeny were generated from the crosses W182BN(CA) X [W182BN(CA) X VA20(CA)] and B73(S) X [B73/S X VA20(CA)] respectively. A linkage test of the *Rf3-KY21* and *Rf-VA20* loci was performed using 76 plants from the three-way-cross W182BN(CA) X [VA20(CA) X KY21(S)].

Male fertility

The ability to shed pollen was recorded for all plants. Shed pollen was collected in acetocarmine stain (Rhoades and Dempsey, 1966) and examined at 100X magnification. Samplings of 250-500 pollen grains from each plant were classified as either normal (starch-filled) or aborted. Data are presented as percent starch-filled grains.

Preparation of genomic DNA and Southern hybridization

Genomic DNA was isolated from leaf tissue (Dellaporta et al., 1983), digested with restriction endonucleases, fractionated by electrophoresis through 0.8% agarose gels, and blotted onto a nylon membrane support (Hybond^{TM}-N Amersham) according to previously described procedures (Southern, 1975). Hybridizations were performed according to the protocol described by Church and Gilbert (1984). Membranes, which were hybridized a minimum of 16 hours, were washed 15 minutes 3 times in 1% SDS and .04 M Na_2HPO_4 pH7.2 at 65°C. Membranes were exposed to X-OMAT^{TM} AR film (Kodak) with an intensifying screen at -80°C for 2-8 days. Re-used membranes were stripped of probes between each hybridization by submersion in a 100°C solution of 0.1% SDS, followed by cooling to room temperature. The *npi271*, *npi297*, *npi122*, *npi456*, and *npi298* clones were provided by Native Plants Inc. (currently available through Pioneer HiBred Inc.). Dr. B. Burr (Brookhaven National Lab) supplied the *bnl12.09* and *bnl17.14 (pio200075)* clones. The *c2* and *whp* clones were obtained from Dr. U. Weinand (Max-Planck-Institut). The *vp1* clone and the *wx1* clone were obtained from Dr. D. R. McCarty (U. of Florida) and Dr. S. Wessler (U. of Georgia) respectively. Probes were radiolabeled by randomly primed DNA synthesis in the presence of alpha-^{32}PdCTP (Feinberg and Vogelstein, 1983). Labeled probe and unincorporated nucleotides were separated either chromatographically through a G-50-50 Sephadex column or by precipitation in 1 volume isopropanol and 1/5 volume 5M ammonium acetate.

Results

Mapping the Rf3 locus

Heterozygous semi-fertile F1s were used to pollinate a W182BN(CA) inbred to generate the BC1KY21^{W182BN} progeny. Based upon gametophytic restoration, all BC1 progeny should be male semi-fertile. Unexpected male-steriles were observed in all BC1 populations (Table 3-1). Only semi-fertile BC1 progeny were used in RFLP linkage experiments. The nature of the unexpected male-steriles will be discussed below.

Linkage to the *Rf3-KY21* restoring allele was tested by probing DNA from 47 BC1KY21^{W182BN} plants with 6 previously mapped chromosome 2 cDNA and genomic

Table 3-1. Occurrence of unexpected male sterile plants (no shed or < 25% normal pollen grains) and the male fertile plant phenotype in BC1 mapping populations.

Population	Number of Male Steriles	Number of Male Fertiles
W182BN(CA) X [W182BN(CA) X VA20(CA)] {BC1VA20^{W182BN}}	53	113
W182BN(CA) X [W182BN(CA) X KY21(S)] {BC1KY21^{W182BN}}	4	47
W182BN(CA) X [W182BN(CA) X CE1(VG)] {BC1CE1^{W182BN}}	7	39
B73(S) X [B73(S) X VA20(CA)] {BC1VA20^{B73}}	6	79

clones. The *bnl12.09*, *npi271*, and the *npi456* clone were unlinked to fertility restoration with 28, 28, and 27 recombinants, respectively. Markers linked to fertility restoration were *npi298*, *whp* and *bnl17.14* which had 10, 3 and 3 recombinant progeny respectively. Double recombinants were detected between *npi12.09* and *whp* (6); *npi456* and *bnl17.14* (1); and *npi271* and *npi298* (3) (Figure 3-1). Gene order and linkage estimates among all loci were determined by three-point mapping analysis. Figure 3-2 summarizes these results. The proximal *whp* and distal *bnl17.14* markers, each an estimated 6.4 cM distance from CMS-S fertility restoration, were used in all subsequent *Rf3* linkage experiments.

The DNA from the unexpected male sterile plants in the BC1KY21^{W182BN} population were hybridized with the 2L RFLP clones as well as the *vp1* and *wx1* clones which are located on chromosomes 3 and 9 repectively. Only the *rf3* associated alleles were transmitted for the six 2L markers tested (data not show). Three progeny showed *vp1* RFLP fragments and two progeny showed *wx1* RFLP fragments from both parental genomes (Figure 3-3).

The identification of RFLP loci linked to *Rf3* was also attempted using the inbred VA20(CA) as the source for the CMS-S restorer gene. DNA samples from 19 BC1VA20^{W182BN} semi-fertile plants were probed with *npi271*, *npi297*, *npi122*, *npi456*, *npi298*, *c-2/whp* and *bnl17.14*. No linkage was detected between these probes and CMS-S fertility restoration (Table 3-2). DNA from 18 additional BC1VA20^{W182BN} and40 BC1VA20^{B73} progeny when probed with the *whp* and *bnl17.14* clones, confirmed independent assortment of the *Rf-VA20* restoring locus and the above 2L

Figure 3-1. Autoradiograph of *Sst*I digested genomic DNA hybridized with the *c2* cDNA clone. The 2L *whp* locus was associated with the 10.2 kb (a) and 5.3 kb (b) fragments. The duplicate *c2* locus on 4L did not segregate (data not shown). Recombinants were identified by the loss of the KY21 10.2 kb fragment.

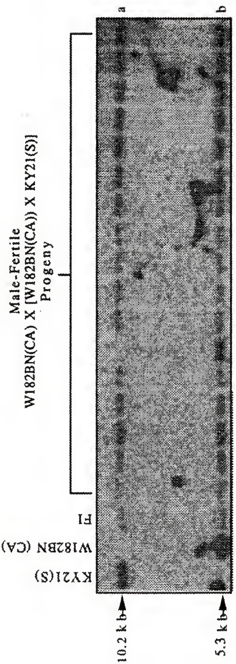

Male-Fertile
W182BN(CA) X [W182BN(CA)) X KY21(S)]
Progeny
KY21(S)
W182BN (CA)
FI
a
b
10.2 k b
5.3 k b

Figure 3-2. Partial map of maize chromosome 2 showing linkage of RFLPs and restoration of fertility by the gene, *Rf3*. Map distances are presented in cM and were estimated from the number of recombinants indentified within a family of 47 $\mathrm{BC1KY21}^{\mathrm{W182BN}}$ plants. Only the *bnl12.09* locus has been predicted to map to 2S, the remaining 5 RFLP markers have been mapped to 2L.

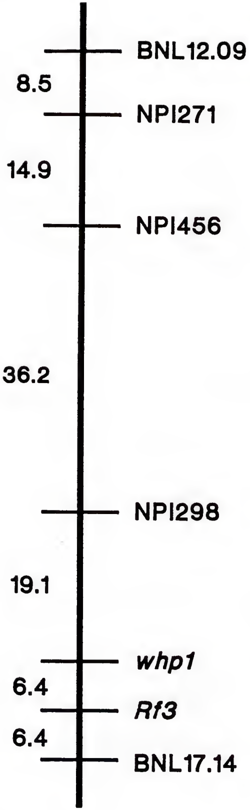

BNL12.09
8.5
NPI271
14.9
NPI456
36.2
NPI298
19.1
whp1
6.4
Rf3
6.4
BNL17.14

Figure 3-3. Southern blot analysis showing the four unexpected male sterile BC1KY21^{W182BN} progeny resulted from a pollination by a F1 parent heterozyous for the KY21 and W182BN genomes.

Panel A. Genomic DNA digested with the restriction endonuclease *Hin*dIII and hybridized with the *vp1* cDNA clone

Panel B. Genomic DNA digested with the restriction endonuclease *Eco*RI and hybridized with the *wx1* cDNA clone

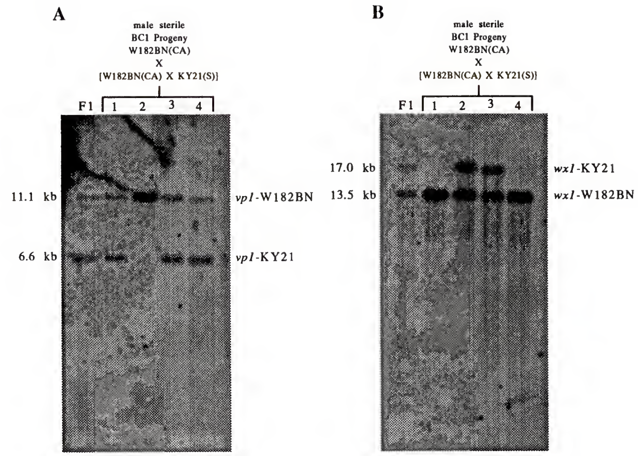

A
male sterile
BC1 Progeny
W182BN(CA)
X
[W182BN(CA) X KY21(S)]
F1
1
2
3
4
11.1 kb
6.6 kb
vp1-W182BN
vp1-KY21
B
male sterile
BC1 Progeny
W182BN(CA)
X
[W182BN(CA) X KY21(S)]
F1
1
2
3
4
17.0 kb
13.5 kb
wx1-KY21
wx1-W182BN

Table 3-2. Linkage test for *Rf-VA20*. Recombination between 2L RFLP markers and plant male fertility phenotypes are presented as a percent of the population size tested, given in parenthesis.

	RFLP probes						
Population	*npi271*	*npi297*	*npi122*	*npi456*	*npi298*	*whp*	*bnl17.14*
BC1VA20^{W182BN}	42% (19)	63% (19)	53% (19)	61% (18)	42% (19)	54% (37)	48% (33)
Male Sterile BC1VA20^{W182BN}						70% (20)	65% (20)
BC1VA20^{B73}						44% (43)	49% (43)

molecular markers. Furthermore, no linkage was detected between the unexpected male sterile phenotype of 20 $BC1VA20^{W182BN}$ plants and the *whp* or *bnl17.14* loci. Subsequently, a direct test for linkage between the standard *Rf3-KY21* and *Rf-VA20* loci was conducted. Male fertile VA20(CA) X KY21(S) hybrids were used to pollinate W182BN(CA). All progeny were classified for male fertility based upon their ability to shed pollen. Microscopic examination of pollen from 45 sampled plants demonstrated the expected semi-fertile phenotype(s). One additional plant displayed a male sterile phenotype (Table 3-3). DNA samples from 75 semi-fertile progeny were probed with the flanking linked *Rf3-KY21* markers, *whp* and *bnl17.14*. Each marker segregated 2 KY21(S) : 1 VA20(CA) allele (Table 3.4).

Descriptive Characteristics of the VA20 Restorer

CMS-S restoration by VA20(CA), KY21(S) and CE1(VG) was compared in F1 and BC1 populations generated with four male sterile inbreds; W182BN(CA), WF9(S), NYD410(CA) and PA884p(CA) (BC1 populations with CE1(VG) were available only in combination with W182BN(CA)). Linkage analysis, the ability to restore different CMS-S strains, and fertility of the pollen populations indicated that the VA20 restorer is distinct from the standard *Rf3* gene.

Male fertility, expressed as percent normal pollen grains is presented in Table 3-3. VA20 populations exhibited a wider range of fertility restoration than the populations carrying the restorer from KY21(S) or CE1(VG) sources.

Table 3-3. Genotype combinations and male-fertility composition of populations used to compare restoration by *Rf* genes from KY21(S), CE1(VG), and VA20 maize strains.

Genotype	Total No. of Plants	No. Male Sterile Plants	No. Male Fertile Plants	% Stained Pollen	
				Average	Range
VA20(CA)	53	0	53	84	46-99
KY21(S)	27	0	27	95	78-99
CE1(VG)	10	0	10	79	64-89
W182BN(CA) X VA20(CA)	43	0	43	61	33-83
W182BN(CA) X KY21(S)	14	0	14	60	54-68
W182BN(CA) X CE1(VG)	6	0	6	50	45-65
NYD410(CA) X VA20(CA)	4	2	2	51	42-87
NYD410(CA) X VA20(CA)	17	16	1	--	--*
NYD410(CA) X VA20(B)	9	9	0	N/A	N/A**
NYD410(CA) X VA20(TC)	6	6	0	N/A	N/A
NYD410(CA) X KY21(S)	4	0	4	--	--
NYD410(CA) X CE1(VG)	23	3	20	--	--
WF9(S) X VA20(CA)	31	1	30	--	--
WF9(S) X KY21(S)	5	0	5	--	--
WF9(S) X CE1(VG)	9	0	9	--	--
PA884p(CA) X VA20(CA)	26	0	26	--	--
PA884p(CA) X KY21(S)	14	0	14	--	--
B73(S) X VA20(CA)	12	0	12	71	39-89
VA20(CA) X KY21(S)	12	0	12	94	85-99

Table 3-3 -- continued

Genotype	Total No. of Plants	No. Male Sterile Plants	No. Male Fertile Plants	% Stained Pollen	
				Average	Range
BC1VA20^{W182BN}	168	55	113	49	25-82
BC1KY21^{W128BN}	51	4	47	50	39-65
BC1CE1^{W182BN}	46	10	36	48	32-63
BC1KY21^{NYD410}	48	12	36	--	--
BC1VA20^{WF9}	27	14	13	--	--
BC1KY21^{WF9}	51	3	48	--	--
BC1VA20^{PA884p}	34	5	29	53	25-76
BC1VA20^{PA884p}	19	6	3	--	--
BC1KY21^{PA884p}	7	0	7	--	--
BC1VA20^{B73}	45	5	40	--	--
BC1VA20^{B73}	40	2	38	--	--
W182BN(CA) X [VA20(CA) X KY21(S)]	45	1	44	63	31-90
W182BN(CA) X [VA20(CA) X KY21(S)]	30	0	30	--	--

*No data collected
**Not applicable

Table 3-4. Linkage test between *Rf3-KY21* and the *Rf* gene inherited from the VA20(CA) genome. Seventy-six progeny from the test cross W182BN(CA) X [VA20(CA) X KY21(S)] were assayed for linkage with *Rf3*-linked RFLPs.

RFLP	KY21(S) Allele	VA20(CA) Allele	X^2 2 : 1	*P*
whp	55	21	0.95	> 0.50
bnl17.14	52	24	0.06	> 0.90

Differential fertility restoration capabilities were first detected in crosses with the male sterile inbred NYD410(CA). Most of the F1 progeny generated with VA20(CA) were male sterile. In contrast, restoration by *Rf3-KY21* appeared normal and only 3 male sterile plants (13%) were observed when the CE1(VG) allele was utilized. Male steriles from NYD410(CA) hybridized with 2 additional VA20 isolines also distinguished restoration by *Rf-VA20* relative to that of the KY21 and CE1 restorers. With the exception of 1 WF9(S) X VA20(CA) plant, male sterile F1s did not occur in combinations of VA20 with the remaining 3 CMS inbred lines. Differences in restoration capabilities were also evident in BC1 progeny. All BC1 populations except PA884p(CA) X [PA884p(CA) X KY21(S)] produced unexpected male sterile plants. Lack of male steriles in the latter population may be due to the small number of plants examined. Comparisons among the remaining BC1 populations showed a trend in the percent of male sterile plants. Combinations with VA20(CA) consistently produced the highest percent of male sterile progeny and combinations with KY21 the lowest percent of male sterile progeny. The one CE1(VG) BC1 population contained an intermediate percentage of male sterile plants. These trends correlated with the range in pollen fertility in that, VA20(CA) combinations showed the most variability, KY21(S) the least and CE1(VG) was intermediate.

Discussion

The KY21 inbred carries the standard *Rf3 (Rf3-KY21)* gene which first identified the gametophytic system of restoration for the maize S-type male-sterile inducing cytoplasm (Buchert, 1961). Cytogenetic data had indicated *Rf3* was proximally located to the 2L.80 breakpoint in the long arm of the chromosome 2 (Laughnan and Gabay, 1978). I generated a BC1 population and performed Southern analysis with previously mapped loci (Burr and Burr, 1991; Franken et al., 1991) to more accurately estimate the relative position of *Rf3-KY21*.

Recombination among RFLP markers and male-fertility restoration of W182BN(CA) X [W182BN/CA X KY21(S)] progeny showed the proximal to distal linkage order *bnl12.09*, *npi271*, *npi456*, *npi298*, *whp*, and *bnl17.14* to be consistent with the maize universal genetic map presented in the 1993 Maize Genetics Cooperation Newsletter (MNL 67:158). Furthermore, the *Rf3-KY21* locus was tightly linked to proximal and distal flanking markers *whp* and *bnl17.14* respectively. This position is in agreement with cytogenetic data which indicated *Rf3* is in the 2L linkage group (Laughnan and Gabay, 1978). *Rf3* is, however, apparently distal rather than proximal to the T2.80 breakpoint as previously reported. The distal placement was suggested by the chromosome 2 map (MNL 67:158) and reported recombinants with In2a.

Four phenotypically male-sterile plants were identified among the BC1KY21^{W182BN} progeny. The possibilities that these were misidentified male steriles or escapes were examined with RFLP linkage analysis. Linkage analysis indicated that the four male-sterile plants resulted from recombinations between *Rf3* and each of 2L RFLP loci tested (data not shown). This is highly unlikely since it requires a recombinational event to occur between the *whp* and *Rf3* loci and a second one to occur between the *Rf3* and *bnl17.14* loci. Additional evidence that the nonrestoring *rf3* allele was transmitted through pollination by the semi-fertile F1 parent was obtained by testing the transmission of other unlinked RFLPs. Combined results from Southern analysis with the *wx1* and *vp1* loci showed that the male sterile BC1KY21^{W182BN} plants were generated with pollen from a F1 plant heterozygous for the KY21 and W182BN genomes and were not from contaminant pollinations by the W182BN(LF) maintainer line (Figure 3-3).

Presence of a CMS-S *Rf* gene in the VA20 genome was shown when CMS-S male sterile inbreds W182BN(CA) and B73(S) crossed with VA20(CA) were restored to semi-fertility. Preliminary data from BC1VA20^{W182BN} progeny did not detect linkage between chromosome 2 RFLP loci and male fertility as expected, suggesting the VA20 restorer gene (*Rf-VA20*) was not allelic to *Rf3-KY21*. Linkage between *Rf-VA20* and *Rf3-KY21* was subsequently tested using the three-way-cross W182BN(CA) X [VA20(CA) X KY21(S)]. Progeny DNA probed with the *Rf3-KY21* linked markers, *whp* and *bnl17.14*, segregated 2 KY21 : 1 VA20 alleles. This segregation is indicative of two major unlinked gametophytic restorer genes. In this model the VA20(CA) X KY21(S) F1 hybrids produce 75% viable and 25% nonviable pollen grains. The nonviable pollen is predicted to have the *rf3-KY21/rf3-KY21 rf-VA20/rf-VA20* genotype and would thus constitute a nontransmitting lethal class for the VA20 *whp* and *bnl17.14* alleles among segregating testcross progeny. The typical phenotype expected of these pollen grains would be empty and collapsed. Estimated male fertility for this F1 hybrid, however, was unexpectedly high (Table 3-3). Sampling error may account for some over-estimation as has been seen in a population where pollen sampling techniques were compared (Kamps et al. unpublished). Additionally, pollen abortion may be delayed as a result of hybrid vigor and environmental effects (Briggle, 1957; Edwardson, 1955; Jones, 1956; Josephson et al., 1978; Lee et al., 1980; Vincent and Woolley, 1972) and, consequently, its characteristic morphology would not be clearly observed.

The VA20(CA) X KY21(S) F1 fertility could also be explained if the VA20 inbred carried either a single sporophytic restorer or combinations of the standard *Rf3* restorer with an unlinked gametophytic or sporophytic restorer. A genetic model in which VA20 carries the standard *Rf3* restorer and an unlinked gametophytic restorer predicts that a BC1 population would segregate 1 fertile : 2 semi-fertile and no male sterile progeny, and the three-way-cross population would segregate 1 fertile : 1 semi-fertile progeny as well as 1 KY21 : 1 VA20 *Rf3*-linked RFLPs. The high frequency of male steriles in the BC1VA20^{W182BN} population, the 2 KY21 : 1 VA20 segregation of the *Rf3*-linked RFLPs in the three-way-cross population, and the pollen data for both populations are inconsistant with these predictions. A single sporophytic gene model predicts 1/4 of the BC1 progeny to be male sterile and linked markers to segregate 1 KY21 : 1 VA20 in the three-way-cross population. Again, the 2 KY21 : 1 VA20 segregation of the *Rf3*-linked RFLPs indicated that this model should also be rejected. A possible two-dominant-duplicate gene system in CMS-S maize was reported by Josephson et al. (1978). The F1 (CMS-S male sterile X VA20(CA) semi-fertile phenotype suggests this model is also not an accurate explanation for restoration by the VA20(CA) genome.

CMS *Rf* genes are identified and classified according to the cytoplasms they restore (Beckett, 1971; Briggle, 1957; Buchert, 1961; Duvick, 1956; Duvick, 1965; Josephson et al., 1978; Kheyr-Pour et al., 1981; Laughnan and Gabay-Laughnan, 1983; Shinjyo, 1969) and may subsequently be mapped to a linkage group (Blickenstaff et al., 1958; Burnham, 1982; Duvick et al., 1961; Laughnan and Gabay, 1975; Laughnan and Gabay, 1978; Maan et al., 1984; Sisco, 1991; Snyder and Duvick, 1969). The detection and selection of exceptional male fertile plants in CMS-S populations resulted in the identification of 10 nuclear mutations to which restored male fertility in CMS-S maize. Six of these restorers were shown to have new linkage relationships in the genome (Laughnan and Gabay, 1975).

Frequent observation of unexpected male sterile BC1VA20^{W182BN} progeny gave an early indication that the VA20 CMS-S restoration system was novel. The inability to identify 2L molecular markers linked to male fertility in this population and a subsequent linkage test with *Rf3-KY21*, revealed only that *Rf-VA20* was probably a gametophytic restorer at an unlinked location in the nuclear genome.

Further information regarding *Rf-VA20* may be gained following the identification of its location in the genome. The possibility that the *Rf-VA20* gene arose from a duplication of the *Rf3* locus is suggested by research conducted by

Helentjaris et al. (1988) and Laughnan and Gabay (1978). Among the nuclear mutations to male fertility identified by Laughnan and Gabay (1975) was one which behaved similarly to *Rf3*. This was designated *RfIV* and was later located to chromosome 3 (Laughnan and Gabay, 1978). Results from RFLP analysis in maize by Helentjaris et al. (1988) revealed that at least 29% of the genome is comprised of "duplicate" sequences. The majority of the 2L duplications were located to chromosome 7. Small segments of 2L DNA sequences were also found to be duplicated on the remaining chromosomes, with the exception of chromosomes 1 and 8. Linkage tests, like the one conducted here with *Rf3*, which use the *RfIV* loci, the chromosome 3, and the chromosome 7 duplicated 2L DNA sequences may identify the map position of *Rf-VA20* and provide primary information regarding its origin. Tight linkage between *Rf-VA20* and *RfIV* might indicate a common origin. In addition, the probability that these genes did indeed arise via a duplication mechanism rather than spontaneous mutation or genomic transposition events is increased if tight linkage with 2L RFLP loci duplicated on chromosome 3 are also identified.

Linkage changes together with distinctive restoration properties occur with new CMS restorer genes (Briggle, 1957; Gracen and Grogan, 1974; Laughnan and Gabay, 1973; Laughnan and Gabay, 1975). Genetic tests which compared the restorative properties associated with the KY21(S), CE1(VG) and VA20 genomes were conducted. Both the KY21(S) and CE1(VG) inbred lines were used in these comparative analyses with the *Rf-VA20* gene because their reported allelic *Rf3* genes (Laughnan and Gabay, 1978) provided a test of nuclear background effects for *Rf3* genes.

The differential ability of a nuclear restorer to overcome the effects of sterile inducing cytoplasms has previously been demonstrated (Anandakumar and Subramaniam, 1992; Briggle, 1957; Gracen and Grogan, 1974; Josephson et al., 1978; Raj and Virmani, 1988; Sisco et al., 1985; Vidakovic, 1988). Restoration capabilities were shown to differ between populations generated from hybridization with the VA20 inbreds and both the CE1(VG) and KY21(S) inbreds (Table 3-3). Because CMS fertility restoration by *Rf* genes is generally a qualitatively inherited trait, it is tempting to suggest that the variation observed between *VA20-Rf* and the allelic *Rf3* genes from KY21(S) and CE1(VG) results from a basic functional change. The possibility that the variation is caused by significant genetic interactions between the hybridized nuclear genomes and the cytoplasm cannot be eliminated at this time, however, since isogenic lines with the *VA20-Rf*, *KY21-Rf3* and *CE1-Rf3* genes were not used to generate the respective BC1 populations examined.

In another test using isogenic VA20 lines with the S cytoplasmic subgroups (CA), (B) and (TC), the effect of nuclear interaction on CMS fertility restoration was examined. The *Rf-VA20* gene from each source failed to restore fertility to the NYD410(CA) line. This was unexpected since one of the restored VA20 lines and NYD410 both had the (CA) cytoplasm. The possibility exists that male fertility for the NYD410(CA) X VA20 combinations may occur only in the presence of additional weak restorers or modifiers, the effects of which are often influenced by the environment (Blickenstaff et al., 1958; Briggle, 1957; Josephson et al., 1978; Marshall et al., 1974; Tracy et al., 1991; Vincent and Woolley, 1972). An environmental influence was not clearly indicated in these populations. Poor fertility, defined as few to no exerted anthers and very little to no pollen shed, was observed in NYD410(CA) X VA20 F1s grown under both Florida winter greenhouse and early July field conditions. The expected semi-fertile phenotype, however, resulted when NYD410(CA) was crossed with the KY21(S) and CE1(VG) inbreds carrying the standard *Rf3* gene (Table 3-3).

The deviation detected in percent pollen fertility together with the elevated frequency of unexpected male steriles in BC1VA20 populations relative to BC1KY21 and BC1CE1 populations further implies that the VA20 CMS-S system is distinct and likely more complicated than the standard restoration system involving the *Rf3* gene. Vidakovic (1988) proposed that the CMS-C system is also more complicated than a single gene mechanism hypothesized by Duvick (1972) and the two or three complimentary gene mechanism proposed by Josephson et al. (1978). Similarly, a complex interaction between two independent nuclear restorer genes in combination with various CMS lines has been reported in rice (Anandakumar and Subramaniam, 1992; Raj and Virmani, 1988) and wheat (Sage, 1976). Without further research it is not reasonable to propose a more specific model of *Rf-VA20* fertility restoration in terms of genetic interaction, regulation and the effects of modifying genes on CMS-S fertility restoration by the VA20 genome. That the VA20 genome carries a unique, naturally occurring CMS-S *Rf* gene is indicated by the linkage data combined with the apparent differences in gene action described above.

Skewed male-fertility inheritance (Buchert, 1961) and pollen phenotype (Lee et al., 1980) defined the gametophytic restoration system for maize S-type CMS. These two characteristics have been used extensively in subsequent studies of this system. In this research I have demonstrated several of the many uses for the RFLPs closely linked to *Rf3*. Mapping and "allelism" testing of CMS-S restorers had previously been accomplished through the use of cytological markers and microscopic examination of the male fertile phenotype. That research assigned the standard *Rf3* locus to the 2L linkage group (Laughnan and Gabay, 1978). With RFLP analysis I have defined more precisely the map position of *Rf3-KY21* to be within an estimated 12.8 cM region between the *whp* and *bnl17.14* loci. Use of these markers will lift the imposed limitation of incorporating cytological markers and their ensuing disadvantages into genetic and breeding programs.

In general, the ability to molecularly characterize a genotype offers several advantages in the study of a male fertility trait. Normal and aborted pollen grain percentages can be significantly confounded by external environmental factors, genomic interactions, nuclear male- sterile mutations and pollen sampling procedures. Linked RFLPs can circumvent these factors and provide a direct assay of allelic transmission. The importance of this assay was demonstrated here with the *Rf3-KY21/Rf-VA20* linkage test. Additionally, RFLPs enable a quick and objective determination of the genetic identity of unexpected male-sterile plants with respect to the restorer gene. For example, testing for the *Rf3*-linked alleles allowed suspected *rf3/rf3* plants to be discarded from the linkage and transmission experiments. The *whp* and *bnl17.14* RFLPs can now help clarify and expand *Rf3* research with their capacity for; pre-tassel screening, new transmission experiments, simpler linkage testing, a more direct method to examine *Rf* inheritance anomalies, and a means to confirm insertional inactivation by a transposable element. For reasons discussed above, continued research with the VA20 restoring system should first identify markers linked to its *Rf* locus.

CHAPTER 4
GENETIC DOMINANCE OF THE GAMETOPHYTIC GENE *RF3* DETERMINED BY CHROMOSOME DOUBLING

Introduction

Skewed inheritance and a semi-fertile phenotype demonstrated that fertility restoration to the S-type cytoplasmic male sterile (CMS-S) maize by the *Rf3* gene occurred in the male gametophyte (Buchert, 1961). Traditionally, the normal appearing pollen grains from these plants are considered restored and their designated genotype, *Rf3*, suggests a functional allele. Conversely, aborted pollen grains with a collapsed phenotype are designated *rf3*, a nonfunctional allelic form. This implied dominant-recessive allelic relationship was questionable since the morphological *Rf3* phenotype had only been examined in haploid (1N) pollen (Lee et al., 1980; Laughnan and Gabay-Laughnan, 1983).

To assess a dominant-recessive interaction it is necessary to observe gene expression, as revealed by the phenotype, in the appropriate 2n tissue. The 2n gametic genome normally produced by tetraploid (4n) plants provides a convenient vehicle to directly investigate gametophytically expressed allelic relationships. No maize 4n strains with the S-type male sterile inducing cytoplasm were available. The *Rf3* genotype of available 4n strains was, therefore, also unknown since the *Rf3* phenotypes can only be observed as a consequence of the specific nuclear-cytoplasmic interaction. A strategy was devised to develop 4n plants which would meet both requirements.

Tetraploids and polyploids may be developed using any one of several possible techniques (Curtis and Doyle, 1992; De Haan, et al. 1992; Hermsen, 1984; Ramanna, 1979; Randolf, 1932; Rhodes and Dempsey, 1966). Meiotic mutants have been used successfully for ploidy manipulation in plant breeding programs (Alexander, 1957), cell biology research (Curtis and Doyle, 1991) and basic genetic studies (Nel, 1975). For a maize plant homozygous for the meiotic mutant elongate (*el*), an ear will develop both normally reduced and unreduced female gametes at a variable frequency (Alexander, 1957; Rhoades and Dempsey, 1966). Upon fertilization, these gametes will develop into seed with either a normal or a shriveled endosperm, depending on the ploidy of the male gamete. This is a beneficial characteristic, in that these *el/el* plants are easily identifiable and progeny with the desired ploidy can be selected by the seed phenotype. With regard to ploidy manipulations, the filled seed phenotype requires a genetic balance in the endosperm tissue. The ratio of 2 maternal : 1 paternal genomes in the endosperm tissue achieves this balance (Lin, 1984). This occurs when female gametes are fertilized by pollen of the same ploidy, and conversely, a shriveled endosperm is expected when the female and male gametes are of different ploidys. A strategy which combined the *el* mutation and maternal inheritance of the cytoplasm generated the necessary CMS-S 4n maize plants for the experiments presented here.

The objectives of this study were to develop a system to genetically assess the dominant-recessive allelic relationship for gametophytically expressed genes, and identify the dominant allele for the fertility restorer gene, *Rf3*. By generating CMS-S 4n maize plants it was possible to demonstrate phenotypically and genetically that the restoring *Rf3* allele was the dominant, i.e. functional, form of the gene.

Materials and Methods

Plant materials

The restored CMS-S line KY21(S) was provided by J. Laughnan and S. Gabay-Laughnan (U. of Illinois). The elongate line (84-666-4) and tetraploid (4n) lines, W23 conversion (W23C) and 85-676-1 were provided by the Maize Genetics Stock Center (U. of Illinois). Tetraploids with the CMS-S type cytoplasm were generated by pollinating F2 plants from selfed progeny of CMS-S KY21 X 84-666-4 with W23C. Maize CMS-S tetraploids were distinguished from 1N X 2N pollinations by seed phenotype; the former were plump and the latter had the characteristically defective and shriveled endosperm (Lin, 1984). Kernel phenotype was used in subsequent crosses with 4n lines to confirm ploidy of progeny derived from the plump seed. CMS-S 4n progeny were grown in the greenhouse, scored for male fertility (as described below) and used as males in crosses with the 4n strain 85-676-1 to test transmission of the *Rf3* alleles through pollen. The genotypes of the parental F2 plants with respect to *Rf3* were determined by microscopic examination of pollen and Southern blot analysis.

Pollen phenotype

Male fertility was evaluated at 100X magnification of shed pollen collected in an acetocarmine stain (Rhoades & Dempsey, 1966). Approximately 500 pollen grains from each plant were examined and classified as either normal (starch filled) or aborted.

Preparation of genomic DNA and Southern hybridization

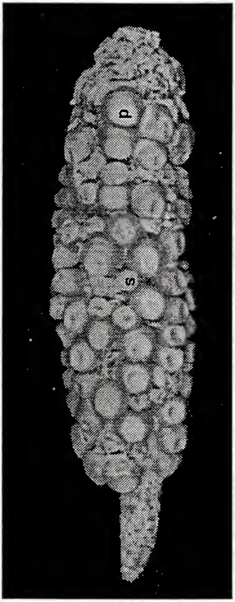
Genomic DNA was isolated from leaf tissue (Dellaporta et al. 1983), digested with restriction endonucleases, fractionated by electrophoresis through 0.8% agarose gels, and blotted onto a nylon membrane support (Hybond^{TM}-N Amersham) according to previously described procedures (Southern, 1975). Hybridization was performed according to the protocol described by Church and Gilbert (1984). Membranes which had hybridized a minimum of 16 hours were washed 3 times in 1% SDS and 0.04 M Na_2HPO_4 pH 7.2 at 65°C. Membranes were exposed to X-OMAT$_{TM}$ AR film (Kodak) with an intensifying screen at -80°C for 2-8 days. Re-used membranes were stripped of probes between each hybridization by submersion in a 100°C solution of 0.1% SDS and allowed to cool to room temperature. The *bnl17.14* probe was provided by B. Burr (Brookhaven National Laboratory, Upton, NY). The *whp* specific probe was provided by U. Weinand (Inst. Allge. Bot., Hamburg 52, Germany). Probes were radiolabeled by randomly primed DNA synthesis in the presence of alpha-^{32}PdCTP (Feinberg and Vogelstein, 1983). Labeled probe and unincorporated nucleotides were separated either chromatographically through a G-50-50 Sephadex column or by precipitation in 1 volume isopropanol and 1/5 volume 5M ammonium acetate.

Results and Discussion

Generation of CMS-S Tetraploid Maize

CMS-S maize tetraploid (4n) plants were generated to investigate the dominant-recessive relationship between the restoring and nonrestoring *Rf3* alleles. The KY21(S) inbred was fertilized with pollen from the maize strain 84-666-4 which carried the meiotic mutant elongate (*el*). Eight KY21(S) X 84-666-4 F1 progeny were grown in the greenhouse. Microscopic examination of shed pollen from these F1 hybrids showed a semi-fertile male phenotype (data not shown) indicating the 84-666-4 line was homozygous for the nonrestoring allele (here on designated $rf3_3$). A self pollinated F1 plant produced a segregating F2 population which ranged from male-sterile to fully fertile (data not shown). Plants homozygous for the *el* mutation were detected by the characteristic production of both normally reduced (1n) and unreduced (2n) female gametes on the same ear (Rhoades and Dempsey, 1966). When fertilized these gametes result in plump and shriveled seeds (Lin, 1984). Both seed types occur on the *el/el* plants because the mutation does not appear to effectively interrupt meiosis in all egg cells (Rhoades and Dempsey, 1966). CMS-S 4n progeny were recovered as plump seed resulting from the fertilization of CMS-S 2n female gametes by 2n pollen from the 4n line W23C (Figure 4-1). Progeny from each of these F2 plants were maintained as the separate families CMS-S: 74, 75, 76 and 11. Tetraploid status of these plump seeds was demonstrated by normal seed development in subsequent crosses with known 4n maize plants (not shown).

Figure 4-1. Ear from the cross CMS-S F2 (*el/el*) X W23C (4n) The plump seed phenotype (p) is indicative of fertilization of an unreduced female gamete (2n) by the 2n pollen from W23C. Shrunken seeds (s) occurred when haploid (1n) egg cells from normal meiotic division were fertilized by the 2n pollen (Lin, 1984).



p
s

Parental F2 plants of the CMS-S 74, 75, 76 and 11 families were characterized for male fertility and *Rf3*-linked RFLPs. F2 plants which gave rise to the CMS-S 74 and 75 families shed more than 80% stainable pollen and carried only RFLP alleles associated with the KY21(S) inbred (Table 4-1). These observations were consistent with a homozygous restored, *Rf3/Rf3*, genotype. The CMS-S 74 and 75 4n progeny were, therefore, expected to carry 2 copies of a known restoring *Rf3* allele (designated *KY21-Rf3*).

The theoretical percent stainable pollen values for CMS-S 4n plants are presented in Table 4-2. Expected stainable pollen from plants with the *KY21-Rf3/KY21-Rf3/rf3/rf3* genotype was either 83-100%, or conversely, 0-17% depending on which *Rf3* allele was the functional form. Progeny from the CMS-S 74 and CMS-S 75 families developed large tassels with fully exerted anthers. The average observed stainable pollen shed by these families was 94% for the former and 86% for the latter (Table 4-3). Both families resulted from pollination by the same W23C plant. The known presence of 2 *KY21-Rf3* alleles and the high level of apparent male fertility were consistent with a hypothesis that the restoring allele, *Rf3*, is dominant relative to the nonrestoring allele, *rf3*.

Male fertility estimates for the parental F2 plants of the CMS-S 76 and CMS-S 11 families were 52% and 54%, respectively. Southern blot analysis showed the *Rf3*-linked *whp* and *bnl17.14* clones each hybridized to 2 DNA restriction fragments (Table 4-1). The *whp* clone hybridized to 13.2 kb and 17.8 kb *Bam*H1 DNA fragments, and the *bnl17.14* clone hybridized to 20.0 kb and 7.2 kb *Eco*RV DNA fragments. These

Table 4-1. Genotypes of the F2 plants which produced the 4 CMS-S tetraploid (4n) families. Pollen data is presented as an estimate from a possible 100% starch filled, stainable pollen. Each X represents a single copy of the RFLP allele. The *whp* and the *bnl17.14* hybridized genomic DNA were digested with *Bam*H1 and *Eco*RV restriction endonucleases, respectively.

		whp		bnl17.14	
F2 Plant	Stained Pollen Grains	KY21 *Rf3-KY21* 13.2 kb	elongate $rf3_3$ 17.8 kb	KY21 *Rf3-KY21* 20.0 kb	elongate $rf3_3$ 7.2 kb
54-58	84%	XX		XX	
54-36	88%	XX		XX	
54-22	52%	X	X	X	X
54-52	54%	X	X	X	X

Table 4-2 Predicted male-fertile phenotypes of CMS-S 4n maize plants dependant on the dominant-recessive relationship between *Rf3* alleles. *Rf3* represents the restoring allele and *rf3* represents the nonrestoring allele.

	% Stainable Pollen	
Plant genotype	*Rf3* dominant	*rf3* dominant
Rf3/Rf3/Rf3/Rf3	100	male-sterile
Rf3/Rf3/Rf3/rf3	100	50
Rf3/Rf3/rf3/rf3	83	17
Rf3/rf3/rf3/rf3	50	male-sterile
rf3/rf3/rf3/rf3	male-sterile	male-sterile

Table 4-3. Proposed genotypes of the CMS-S 4n families. Genotypes of the female and male gametes are presented from left to right with a space between. A mean fertiltiy estimate was calculated for the families derived from homozygous *Rf3-KY21* F2 plants. The formula for this calculation was: % stainable pollen/number of obs. The CMS-S 76 and CMS-S 11 families originated from *Rf3-KY21* heterozygous F2 plants, resulting in a mixture of egg genotypes. Consequently, the male fertile phenotype reflects variable *Rf3* contributions from both parental genomes. The fertility estimate shows only the range of stainable pollen observed.

4n Family	Number of Observations	Fertility Estimate	Proposed Genotype(s)
CMS-S 74	15	94%	*Rf3/Rf3 rf3/rf3*
CMS-S 75	6	86%	*Rf3/Rf3 rf3/rf3*
CMS-S 76	17	99% ↓ 48%	*Rf3/Rf3 Rf3/rf3* *Rf3/Rf3 rf3/rf3* *Rf3/rf3 rf3/rf3*
CMS-S 11	58	97% ↓ ↓ male sterile	*Rf3/Rf3 Rf3/rf3* *Rf3/Rf3 rf3/rf3* *Rf3/rf3 rf3/rf3* *rf3/rf3 rf3/rf3*

correlated with the *KY21-Rf3* restoring and the $rf3_3$ nonrestoring alleles, respectively (Figure 4-2). In the production of the CMS-S 4n plants these alleles were seen to segregate (Table 4-4). The one CMS-S 76 plant examined carried 1 KY21 and 1 elongate associated allele; the CMS-S 11 progeny carried a combination of either 0:2, 1:1 or 2:0 copies of the *whp* alleles from the KY21 and elongate parents. The frequency of these progeny were 3, 8 and 2 respectively. Both fertility estimates and Southern blot analysis demonstrated a heterozygous *Rf3* genotype (*KY21-Rf3*/$rf3_3$) for these F2 parents (Table 4.1).

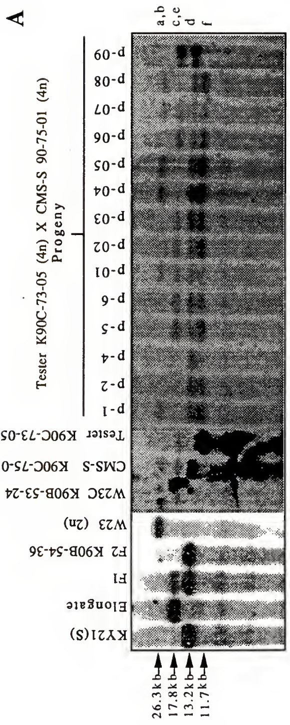
As a consequence of the *el* mutation these plants may yield *KY21-Rf3/KY21-Rf3*, $rf3_3/rf3_3$, and *KY21-Rf3*/$rf3_3$ female gametes. Consistent with this prediction, the CMS-S 76 and 11 families were composed of plants varying in male-fertility. The 17 progeny observed in the CMS-S 76 family developed large, normal appearing tassels, however, shed pollen ranged from 48-99% starch filled grains (Table 4-3). The CMS-S 11 family was observed to be the most variable in both tassel phenotype and apparent male fertility. Plants developed tassels which ranged in appearance from the classical male-sterile phenotype, barren to few exerted and shriveled anthers, to a normal fertile appearance where full tassels of exerted anthers shed heavily. Stainable pollen analysis yielded 4 fertile, 4 male-sterile and 50 plants ranging in fertility from 43-88% (Table 4-3).

The fertile and male-sterile plants likely originated from 2n eggs produced by a second division failure during an abnormal meiosis. Genotypically, these eggs would be *KY21-Rf3/KY21-Rf3* for the former and $rf3_3/rf3_3$ for the latter phenotype and should

Figure 4-2. Southern analysis showing the genotypes and transmission of *Rf3*-linked RFLP alleles (Chapter 3). *Rf3* allelic designations are: a for *rf3* ; b for *Rf3-W23C*; c for *rf3* ; and d for *Rf3-KY21*. Alleles associated with the Tester (4n) parent are designated e and f.

A) *Bam*H1 digested genomic DNA hybridized with the *whp* marker, which was located proximally to the *Rf3* locus.

B) *Eco*RV digested genomic DNA hybridized with *bnl17.14*, the marker located distally to *Rf3*.


A
Tester K90C-73-05 (4n) X CMS-S 90-75-01 (4n)
Progeny
p-1
p-2
p-4
p-5
p-6
p-01
p-02
p-03
p-04
p-05
p-06
p-07
p-08
p-09
Tester K90C-73-05 (4n)
CMS-S K90C-75-01 (4n)
W23C K90B-53-24 (4n)
W23 (2n)
F2 K90B-54-36
F1
Elongate
KY21(S)
a,b
c,e
d
f
26.3kb
17.8kb
13.2kb
11.7kb

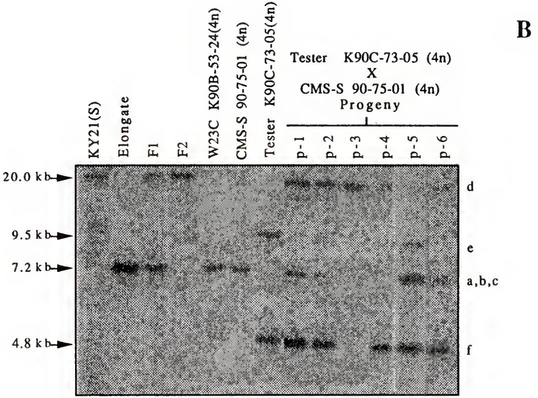


Figure 4-2 -- continued

Table 4-4. Inheritance of *Rf3* associated alleles for CMS-S 4n plants generated from the cross F2 X W23C. Allelic dosages for assayed progeny were determined from the assumption that each parent contributed 2 RFLP alleles to each progeny. The percent stained pollen reflects male-fertility.

		whp			*bnl17.14*		
Plant	Stained Pollen	*Rf3-KY21* (13.2 kb)	elongate W23C (17.8 kb)	W23C (26.3 kb)	*Rf3-KY21* (20.0 kb)	elongate W23C (7.2 kb)	W23C (4.8 kb)
74-03	94%	-*	-	-	2	1	1
75-01	82%	2	1	1	2	2	-
76-01	48%	-	-	-	1	3	0
11-04	10%	0	2	2	-	-	-
11-03	56%	0	3	1	-	-	-
11-09	53%	1	1	2	-	-	-
11-40	54%	1	2	1	-	-	-
11-22	55%	0	2	2	-	-	-
11-25	59%	1	1	2	-	-	-
11-17	66%	2	1	1	-	-	-
11-53	66%	1	2	1	-	-	-
11-42	67%	1	2	1	-	-	-
11-51	67%	1	1	2	-	-	-
11-46	70%	1	1	2	-	-	-
11-13	80%	1	1	2	-	-	-
11-54	80%	2	0	2	-	-	-

* (-) designation for no data

occur with equal frequency. These gametes occurred at a low frequency, which was probably a consequence of the requirements for their production. A low frequency may also explain the lack of male-sterile progeny among the relatively few CMS-S 76 progeny examined. The *KY21-Rf3/rf3*$_3$ eggs may have originated from either a failed reductional or equational division (Nel, 1975). A reductional division failure may only produce the heterozygous female gametes, whereas an equational division failure must also be accompanied by a recombinational event to produce heterozygotes.

Genotype of the W23C Parental Line

The previous analysis showed that the genotype of the 2n female gametes could be directly ascertained from parental male-fertility data combined with the inheritance data of *Rf3*-linked RFLPs. This strategy was not possible, however, for the W23C male gametes. W23C is a converted 4n version of the maize inbred W23 which does not restore fertility to maize plants with the S-type cytoplasm. Because it has a normal cytoplasm and presumably was converted with an elongate line, the genotype with respect to *Rf3* was unknown. Multiple alleles were indicated in the W23C line by the occurrence of 2 hybridized DNA fragments each for the *Rf3*-flanking RFLP loci, *whp* and *bnl17.14* (Figure 4-2).

The progenitor inbred, W23 (2n), and W23C (4n) each carried a 26.3 kb *Bam*H1 fragment which hybridized with the *whp* probe (Figure 4-2A). This indicated the presence of at least 1 nonrestoring *rf3* allele (hereafter referred to as *rf3*$_1$). The identification of male-steriles among the CMS-S 11 progeny supported this conclusion.

The *whp* clone also hybridized to a 17.8 kb *Bam*H1 fragment which was common to W23C, the elongate parental line, and the 85-676-1 4n strain (K90C-73; hereafter referred to as Tester) used to test pollen transmission of the RFLPs associated with the *Rf3* alleles (Figure 4-2A). The *bnl17.14* fragments were polymorphic between W23 and W23C for the 5 restriction enzymes tested (data not shown), however, both W23C and the elongate line carried a 7.2 kb *Eco*RV fragment. The 4.8 kb fragment was common to W23C and the Tester line (Figure 4-2B).

Fertility data suggested that the W23C plants used in generating the CMS-S 4n plants were limited to carrying a single restoring allele (designated *Rf3-W23C*) in addition to the nonrestoring alleles. The majority of CMS-S 74 and 75 progeny would be expected to shed all normal pollen if 2 or more restoring alleles were present in the W23C parent. Instead it was possible to assign progeny to fertile and nearly fertile classes (data not shown). Although a significant difference in average male-fertility was observed between the CMS-S 74 and 75 families, this was likely due to differences in the female parents since both were fertilized with the same male.

Male fertility expectations in combination with Southern analysis of the CMS-S 11 and 76 progeny were also consistent with the proposed *Rf3-W23C/rf3/rf3/rf3* W23C genotype. A transmission test of the *whp* RFLPs to 13 CMS-S 11 progeny provided additional evidence of the single restorer allele carried by the W23C plants used in this study (Table 4-4). Two partially male-fertile plants, 11-03 and 11-22, did not carry the *whp* RFLP associated with the *KY21-Rf3* allele. The percentage of stainable pollen, however, implied a genotype with a single restoring allele. This allele was probably contributed by the W23C parent. The restorer associated *whp* RFLP was absent from the 11-04 plant which was phenotypically male-sterile. Presumably this plant did not receive a restorer allele(s) from either parent. Estimates greater than 65% stainable pollen in the CMS-S 11 family may be attributed to the *KY21-Rf3/KY21-Rf3/_/_* genotype or any combination of 2 restoring *Rf3* alleles. The CMS-S 11-17 phenotype was consistent with the expected male-fertility for a CMS-S 4n plant carrying 2 copies of *KY21-Rf3* (Table 4-2). The single *KY21-Rf3* allele detected in the 71-86% male fertile plants indicated the second restorer required for this phenotype was contributed by the W23C parent. Consistency with the expected male-fertile phenotype for plants with 2 *KY21-Rf3* alleles suggested that the *W23C-Rf3* allele had comparable restoration capabilities. The CMS-S 11-54 plant was assigned 3 restorer alleles, 2 *KY21-Rf3* and 1 *W23C-Rf3*.

The *W23C-Rf3* allele was associated with the *whp* hybridized *Bam*H1 26.3 kb DNA fragment. The association was first indicated by the male- fertility differences observed among the CMS-S 11 progeny which carried 2 doses of this fragment. To illustrate, the 11-04 and 11-22 plants showed identical RFLP phenotypes and lacked the *KY21-Rf3* allele, yet the former was sterile and the later was semi-fertile (Table 4-4). A simple and direct explanation for this result would be that this fragment represented 2 nonpolymorphic alleles, one restoring and one nonrestoring. The *whp* probe also hybridized with a 17.8 kb *Bam*H1 DNA fragment. The W23C source, like the CMS-S F2 source, of this RFLP could be shown to be associated with a nonrestoring allele. The percent stainable pollen shed by the CMS-S 11-17 plant is in agreement with the 2 detected *KY21-Rf3* alleles. Therefore, each allele contributed by W23C in generating the CMS-S 11-17 plant appears to have been nonrestoring. Similarly, the semi-fertile phenotype of the CMS-S 11-40 plant was in agreement with the single detected *KY21-Rf3* allele, and by deduction, the two 17.8 kb RFLPs and the 26.3 kb RFLP were determined to be nonrestoring. The fertility and dosage data for the remaining CMS-11 plants were compatible with these conclusions (Table 4-4).

Assignment of the restoring and nonrestoring *Rf3* alleles to the appropriate *bnl17.14* RFLPs was accomplished using data from the pollen transmission experiment. Stainable shed pollen percentages and RFLP analysis indicated the CMS-S 74-01 and CMS-S 75-03 genomes were comprised of 2 *KY21-Rf3* alleles, and 2 nonrestoring alleles inherited from the W23C paternal parent (Table 4-4). These plants were used to pollinate the Tester 4n plants. Results from Southern hybridizations with the *bnl17.14* probe are presented in Table 4-5. The 20.0 kb *Eco*RV fragment correlated with the *KY21-Rf3* allele. RFLPs originating from the Tester parent were important only in deducing the $rf3_2$ assignment to the 4.8 kb *Eco*RV fragment. The 7.2 kb *Eco*RV fragment could be associated with the $rf3_3$ allele from the elongate parent and allele(s) contributed by the W23C parent (Figure 4-2B). The perceived dosage inheritance and consistency with the *whp* analysis presented above Table 4-4 were the basis for *Rf3* allele assignments to the *bnl17.14* hybridized *Eco*RV fragments (Table 4-5).

A F2 plant heterozygous for the *whp* and *bnl17.14* RFLPs gave rise to the semi-fertile CMS-S 76-01 plant. This phenotype may be genetically described with a

Table 4-5. Southern blot analysis showing the pollen transmitted alleles from CMS-S 74 and CMS-S 75 plants.

	bnl17.14				
	Rf3-KY21	Rf3-W23C rf3$_1$-W23C rf3$_3$-elongate	rf3$_2$-W23C Tester$_1$	Tester$_2$	Proposed Restorer Genotype of the Male Gamete
Plant	20.0 kb	7.2 kb	4.8 kb	9.5 kb	
CMS-S F2	2	0	0	0	*Rf3-KY21/Rf3-KY21**
W23C 53-24	0	3	1	0	*Rf3-W23C/rf3*$_1$*/rf3*$_1$*/rf3*$_2$*
CMS-S 75-01	2	2	0	0	*Rf3-KY21/Rf3-KY21/rf3*$_1$*/rf3*$_1$*
p-1	1	1	2	0	*Rf3-KY21/rf3*$_1$
p-2	2	0	1	1	*Rf3-KY21/Rf3-KY21* or *Rf3-KY21/rf3*$_1$
p-4	2	0	2	0	*Rf3-KY21/Rf3-KY21*
p-5	0	2	1	1	*rf3*$_1$*/rf3*$_1$
p-6	1	1	2	0	*Rf3-KY21/rf3*$_1$

Table 4-5 -- continued

bnl17.14

Plant	Rf3-KY21 20.0 kb	Rf3-W23C rf3$_1$-W23C rf3$_3$-elongate 7.2 kb	rf3$_2$-W23C Tester$_1$ 4.8 kb	Tester$_2$ 9.5 kb	Proposed Restorer Genotype of the Male Gamete
CMS-S F2	2	0	0	0	*Rf3-KY21/Rf3-KY21**
W23C 53-24	0	3	1	0	*Rf3-W23C/rf3*$_1$*/rf3*$_{1/rf31}$*
CMS-S 74-03	2	1	1	0	*Rf3-KY21/Rf3-KY21/rf3*$_1$*/rf3*$_2$*
p-1	1	0	2	1	*Rf3-KY21/rf3*$_2$
p-2	1	0	1	2	*Rf3-KY21/rf3*$_2$
p-3	1	0	2	1	*Rf3-KY21/rf3*$_2$
p-4	1	1	1	1	*Rf3-KY21/rf3*$_1$
p-5	1	1	1	1	*Rf3-KY21/rf3*$_1$
p-6	1	0	2	1	*Rf3-KY21/rf3*$_2$

Note: Progeny from the 4n X CMS-S 4n crosses are designated p-1 to p-6 from both the CMS-S 74 and CMS-S 75 progenitors. Restorer genotype of the progeny refers to the male contribution (i.e. pollen genotype) from fertilization. Allelic dosages were deduced from visual observation combined with the method described in Table 4-4. Genomic DNA was digested with the restriction endonuclease *Eco*RV and hybridized with *bnl17.14*.

* Denotes the restorer genotypes of the parental plants (sporophytic generation)

single restorer allele. Southern hybridization with the *bnl17.14* probe implied the CMS-S 76-01 plant had inherited the *KY21-Rf3* allele. RFLP analysis of the Tester X CMS-S 76-01 progeny, however, did not agree with this supposition. A restorer allele designation for the 20.0 kb fragment would require that 5/11 of these progeny result from fertilization by pollen which did not carry the *bnl17.14* restorer associated RFLPs (Table 4-6). Pollen without major restorer alleles have been shown to effect fertilization (Maan, 1992a). Alternatively, a recombination event between the *Rf3* and *bnl17.14* loci and failure of the 2nd meiotic division is more likely to have occurred during the formation of the unreduced female gamete. This would result in the loss of the *KY21-Rf3* allele. The semi-fertile phenotype of the CMS-S 76-01 plant would then be due to the proposed *W23C-Rf3* allele, a hypothesis that might be confirmed by an analysis with the proximally *Rf3*-linked *whp* RFLPs.

Genetic Demonstration of the Dominant-Recessive Relationship Between *Rf3* Alleles

Rf3 allele assignment to the appropriate RFLPs was necessary to conduct further genetic studies with the CMS-S 4N plants. CMS-S is restored gametophytically by *Rf3*, i.e. male fertility is governed by the *Rf3* genotype of the pollen grain. The ability to genetically demonstrate the dominant-recessive relationship between the restoring and nonrestoring *Rf3* alleles was possible with the 2n male gametes from CMS-S 4n plants and Southern analysis of their progeny. The selected pollinator, CMS-S 75-01, was shown to carry 2 *KY21-Rf3* alleles and 2 nonrestoring alleles from the W23C parent. The former allele was associated with the

Table 4-6. Southern blot analysis showing the pollen transmitted alleles from a CMS-S 76 plant. Two sets of proposed genotypes for the male gametes are presented; those in parenthesis assume no recombination between the *Rf3* and *bnl17.14* loci in the formation of the F2 unreduced female gamete.

			bnl17.14		
	Rf3-KY21 20.0 kb	*Rf3-W23C* $rf3_1$*-W23C* $rf3_3$*-elongate* 7.2 kb	$rf3_2$*-W23C* Tester$_1$ 4.8 kb	Tester$_2$ 9.5 kb	Proposed Restorer Genotype of the Male Gamete
Plant					
CMS-S F2	1	1	-	-	$Rf3\text{-}KY21/rf3_3{}^*$
W23C 53-01	0	3	1	-	$Rf3\text{-}W23C/rf3_1/rf3_1/rf3_2{}^*$
CMS-S 76-01	1	3	0	-	$Rf3\text{-}KY21/rf3_3/rf3_1/rf3_1$
p-1	1	1	1	1	$Rf3\text{-}KY21/rf3_3$ or $Rf3\text{-}KY21/rf3_1$
p-2	1	1	1	1	$Rf3\text{-}KY21/rf3_3$ or $Rf3\text{-}KY21/rf3_1$
p-3	0	2	0	2	$Rf3\text{-}KY21/rf3_3$ or $Rf3\text{-}KY21/rf3_1$ ($rf3_3/rf3_1$; $rf3_1/rf3_1$; or $rf3_3/rf3_3$)
p-4	1	1	1	1	$Rf3\text{-}KY21/rf3_3$ or $Rf3\text{-}KY21/rf3_1$
p-5	2	0	0	2	$Rf3\text{-}KY21/Rf3\text{-}KY21$

Table 4-6 -- continued

Plant	*bnl17.14* *Rf3-KY21* 20.0 kb	 *Rf3-W23C* *rf3*$_1$*-W23C* *rf3*$_3$*-elongate* 7.2 kb	 *rf3*$_2$*-W23C* Tester$_1$ 4.8 kb	 Tester$_2$ 9.5 kb	Proposed Restorer Genotype of the Male Gamete
p-01	1	1	0	2	*Rf3-KY21/rf3*$_1$ or *Rf3-KY21/rf3*$_3$
p-02	1	1	1	1	*Rf3-KY21/rf3*$_3$ or *Rf3-KY21/rf3*$_1$
p-03	0	2	1	1	*Rf3-KY21/rf3*$_3$ or *Rf3-KY21/rf3*$_1$ (*rf3*$_3$*/rf3*$_1$; *rf3*$_1$*/rf3*$_1$; or *rf3*$_3$*/rf3*$_3$)
p-06	0	2	1	1	*Rf3-KY21/rf3*$_3$ or *Rf3-KY21/rf3*$_1$ (*rf3*$_3$*/rf3*$_1$; *rf3*$_1$*/rf3*$_1$; or *rf3*$_3$*/rf3*$_3$)
p-07	0	2	1	1	*Rf3-KY21/rf3*$_3$ or *Rf3-KY21/rf3*$_1$ (*rf3*$_3$*/rf3*$_1$; *rf3*$_1$*/rf3*$_1$; or *rf3*$_3$*/rf3*$_3$)
p-08	0	2	1	1	*Rf3-KY21/rf3*$_3$ or *Rf3-KY21/rf3*$_1$ (*rf3*$_3$*/rf3*$_1$; *rf3*$_1$*/rf3*$_1$; or *rf3*$_3$*/rf3*$_3$)

Note: Progeny from the 4n X CMS-S 4n cross are designated p-1 to p-08. Restorer genotype of these individuals refers to the male contribution (i.e. pollen genotype) from fertilization. Allelic dosages were deduced from visual observation combined with the method described in Table 4-4. Genomic DNA was digested with the restriction endonuclease *Eco*RV and hybridized with *bnl17.14*.

* Denotes the restorer genotypes of the parental plants (sporophytic generation).

whp 13.2 kb and *bnl17.14* 20.0 kb RFLPs and the later allele(s) were associated with the *whp* 26.3 kb (*rf3*$_1$) and 17.8 kb (*rf3*$_2$) RFLPs and the *bnl17.14* 7.2 kb (*rf3*$_1$) RFLP. The genotypes and expected frequencies of male gametes from a 4n CMS-S plant with a *Rf3/Rf3/rf3/rf3* genotype are presented in Table 4-2. A total of 28 progeny were characterized for *whp* RFLPs (Table 4-7). The RFLP phenotype showed 2n male gametes transmitted 1-2 copies of the *KY21-Rf3* associated *whp* allele to all tested progeny. The derivation of allelic dosage, as described above, indicated 6 of these gametes could be assigned the *KY21-Rf3/KY21-Rf3* genotype. The remaining 22 progeny appeared to carry 1 dose of the *KY21-Rf3* allele and 1 dose of either the *rf3*$_2$ or *rf3*$_1$ allele, each resulting from fertilization by a heterozygous (*KY21-Rf3/rf3*$_2$ or *KY21-Rf3/rf3*$_1$) pollen grain (Table 4-7). The observation that the 13.2 kb *whp* RFLP was inherited by all assayed progeny suggested that the restoring form of the *Rf3* gene is the dominant, i.e. functional form. Gene frequencies of the male gametes which generated the 28 test cross plants were 6 *KY21-Rf3/KY21-Rf3* : 22 *KY21-Rf3/rf3*$_x$. This ratio is compatible with the expected 1 *Rf3/Rf3* : 4 *Rf3/rf3* gametic gene frequencies for the dominant restorer hypothesis.

Of the 28 plants evaluated for *whp* RFLP inheritance, 5 were also tested with the *bnl17.14* RFLPs (Figure 4-2B). Inheritance of the *bnl17.14* RFLPs from the male CMS-S 75-01 plant showed consistency with the dominant restorer hypothesis; 1 plant was the product of a fertilization by a *KY21-Rf3/KY21-Rf3* pollen grain and 2 plants developed from fertilization by *Rf3* heterozygous pollen grains. The p-5 plant was exceptional in that the *KY21-Rf3* associated RFLP was absent. Possible explanations

Table 4-7. Southern blot analysis of the *Bam*H1 digested genomic DNA showing pollen transmission of the CMS-S 75-01 *whp* RFLP alleles. The 26.3 kb allele was carried by both W23C (4n) and the CMS-S nonrestoring progenitor W23.

			whp		
	Rf3-KY21	$rf3_2$-*W23C* $rf3_3$-*elongate* Tester_1	*Rf3-W23C* $rf3_1$-*W23C*	Tester_2	Proposed Restorer Genotype of the Male Gamete
Plant	13.2 kb	17.8 kb	26.3 kb	11.7 kb	
CMS-S F2	2	0	-	-	*Rf3-KY21/Rf3-KY21**
W23C 53-01	0	2	2	-	*Rf3-W23C*/$rf3_1$/$rf3_2$/$rf3_2$* or $rf3_1$/$rf3_1$/$rf3_2$/$rf3_2$*
CMS-S 75-01	2	1	1	-	*Rf3-KY21/Rf3-KY21*/$rf3_2$/$rf3_1$
p-1	1	0	1	2	*Rf3-KY21*/$rf31_1$
p-2	2	0	0	2	*Rf3-KY21/Rf3-KY21*
p-4	2	0	0	2	*Rf3-KY21/Rf3-KY21*
p-5	1	1	0	2	*Rf3-KY21*/$rf3_2$
p-6	2	1	0	1	*Rf3-KY21/Rf3-KY21*
p-01	1	1	1	1	*Rf3-KY21*/$rf3_1$
p-02	1	1	0	2	*Rf3-KY21*/$rf3_2$
p-03	2	1	0	1	*Rf3-KY21/Rf3-KY21*
p-04	1	0	1	2	*Rf3-KY21*/$rf3_1$
p-05	1	0	1	2	*Rf3-KY21*/$rf3_1$
p-06	1	1	0	2	*Rf3-KY21*/$rf3_2$
p-07	1	1	1	1	*Rf3-KY21*/$rf3_1$
p-08	1	0	1	2	*Rf3-KY21*/$rf3_1$
p-09	2	2	0	0	*Rf3-KY21/Rf3-KY21*

Table 4-7 -- continued

Plant	*Rf3-KY21* 13.2 kb	*whp* *rf3*$_2$*-W23C* *rf3*$_3$*-elongate* Tester$_1$ 17.8 kb	*whp* *Rf3-W23C* *rf3*$_1$*-W23C* 26.3 kb	*whp* Tester$_2$ 11.7 kb	Proposed Restorer Genotype of the Male Gamete
p-10	1	2	0	1	*Rf3-KY21/rf3*$_2$
p-11	1	1	0	2	*Rf3-KY21/rf3*$_2$
p-12	1	2	0	1	*Rf3-KY21/rf3*$_2$
p-13	1	1	1	1	*Rf3-KY21/rf3*$_1$
p-14	1	1	1	1	*Rf3-KY21/rf3*$_1$
p-15	1	2	0	1	*Rf3-KY21/rf3*$_2$
p-16	1	2	0	1	*Rf3-KY21/rf3*$_2$
p-17	1	1	1	1	*Rf3-KY21/rf3*$_1$
p-18	1	1	1	1	*Rf3-KY21/rf3*$_1$
p-19	1	1	0	2	*Rf3-KY21/rf3*$_2$
p-20	2	0	0	2	*Rf3-KY21/Rf3-KY21*
p-21	1	0	1	2	*Rf3-KY21/rf3*$_1$
p-22	1	0	1	2	*Rf3-KY21/rf3*$_1$
p-23	1	0	1	2	*Rf3-KY21/rf3*$_1$

Note: Transmission of the *Rf3*-linked RFLPs through 2n pollen from CMS-S 4n plants. Progeny from the 4n X CMS-S 4n cross are designated p-1 to p-23. Restorer genotype of these individuals refers to the male contribution (i.e. pollen genotype) from fertilization. Allelic dosages were deduced from visual observation combined with the method described in Table 4-4.

* Denotes the restorer genotypes of the parental plants (sporophytic generation).

for this absence may be 1) exceptional pollen with nonrestoring alleles effecting fertilization, or 2) a recombination event between *Rf3* and the distal *bnl17.14* locus during the formation of the unreduced female gamete. The first possibility had previously been reported in wheat (Maan, 1992a). I have also observed this in diploid maize. When the gene order and heterozygosity at the *whp* locus are considered, the latter explanation appears the more likely of the two. This exceptional plant and the ability to deduce the W23C genotype were among the advantages for testing inheritance of both *Rf3*-flanking RFLPs. Lack of Tester *bnl17.14* alleles suggested that the p-3 plant was a contaminant diploid.

Summary

The primary objective of this study was to determine the dominant-recessive relationship between the gametophyically expressed alleles. For the *Rf3* gene this required that both alleles occur together in the appropriate tissue, the male gametes, and in the presence of the S-type sterile inducing cytoplasm. These criteria were met with the development of maize CMS-S 4n plants. Doubling the genome was accomplished with a strategy in which a meiotic mutant was employed. The male gamete population of CMS-S 4n maize plants shown to have inherited 2 copies of the restoring allele, consisted predominately of starch filled grains. The expected genotypic ratio of these gametes was 1 *KY21-Rf3/KY21-Rf3* : 4 *KY-Rf3/rf3*. Using these plants as males in crosses with another maize 4n line genetically tested function of the *Rf3* heterozygous male gametes presumed to be in the stained pollen population.

Rf3 allelic transmission was evaluated by ascertaining the inheritance of the *whp* and *bnl17.14* RFLPs. The *Rf3* and *rf3* alleles were shown to co-transmit through heterozygous pollen. In addition, homozygous and heterozygous allelic frequencies among progeny resulting from these pollinations was in agreement with the hypothesis that the restoring form (*Rf3*) was functionally dominant to the nonrestoring form (*rf3*).

CHAPTER 5
CONCLUSIONS

The purpose of this research was to develop a method to discern the dominant allele, i.e. functional form, of a gametophytically expressed gene. The maize CMS-S fertility restoring gene *Rf3* was selected for the investigations reported here for two primary reasons 1) the phenotype is relatively easy to identify and, 2) the basic information obtained is critical to further investigations on nuclear-cytoplasmic interactions within this system. The required ability to accurately track the inheritance of *Rf3* alleles was met by identifying closely linked RFLP marker loci. Hybridizations with the *whp* and *bnl17.14* DNA clones showed the genomic DNA sampled from semi-fertile BC1KY21^{W182BN} progeny carried single copy homologous sequences. Linkage estimates between these molecular markers and CMS-S fertility restoration were calculated using a three-point analysis with identified recombinant and nonrecombinant progeny. This analysis located *whp* approximately 6.4 cM proximal and *bnl17.14* 6.4 cM distal to the fertility restoring locus. The *whp* and *bnl17.14* RFLPs were used in subsequent experiments designed to determine the functional *Rf3* allele.

The restoring and nonrestoring *Rf3* alleles were manipulated to be naturally expressed together in 2n male gametes from CMS-S 4n maize plants. This was accomplished by doubling the ploidy of maize CMS-S diploid plants using the meiotic mutant *el*. The *Rf3* genotypes of the plants used in these experiments were reflected by the linked *whp* and *bnl17.14* alleles. The correlation between the expressed pollen phenotypes and the genotype of the CMS-S 4n maize plant from which it was shed provided the first evidence that the restoring form of the *Rf3* gene was dominant and the nonrestoring form was not functional.

Conclusive genetic evidence that the restoring allele was the functional form of *Rf3* was obtained by testing the transmissibility of both alleles through 2n pollen from CMS-S 4n maize plants. The various *Rf3* alleles contributed by KY21/S, W23C, elongate (84-666-4) and the tester tetraploid (85-676-1) plants were assigned to hybridized DNA fragments by integrating RFLP and male-fertility data from several populations. The DNA from progeny derived from a testcross between an unrelated 4n maize plant pollinated by a CMS-S 4n maize plant was hybridized with the *Rf3*-linked molecular markers, *whp* and *bnl17.14*. Results from these experiments showed the co-transmission of RFLP alleles linked to the *Rf3* alleles through 2n pollen. Dominance of the restoring form of the *Rf3* gene was thereby demonstrated genetically.

The results presented here showed that doubling the genomic ploidy to determine the functional allele of *Rf3* is an usable approach for other gametophytically expressed genes. During the course of the *Rf3* mapping experiments another CMS-S restorer gene was discovered in the maize VA20 genome. Linkage analysis with the standard *Rf3* gene (designated *Rf3-KY21*) carried by the maize inbred KY21/S showed that this new gene (designated *Rf-VA20*) is gametophytically expressed and not linked to any loci on the maize 2L chromosome. Variation in restoration capabilities compared with *Rf3* genes suggested the possibility that the mechanism by which *Rf-VA20* restores fertility to maize plants with CMS-S cytoplasm may be unique. Additional research including comparisons between isogenic lines with the two restorers and identifying the position of the *Rf-VA20* within the maize genome are needed. Strategies which utilize information regarding maize duplicated DNA sequences and the *RfIV* CMS-S restorer were proposed to initiate the mapping experiments. These investigations could conceivably provide some basic information regarding the origin(s) of other known CMS-S restoring loci.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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